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(54) Title: METHOD TO IDENTIFY TUMOR SUPPRESSOR GENES

(57) Abstract

This invention provides a method of indentifying a tumor suppressor gene of a cell(s) which comprises the following steps: a) obtaining cDNA or mRNA from a normal cell(s); b) preparing cDNA from the cell(s) if mRNA is obtained in step (a); c) preparing a library from said cDNA, wherein the cDNA is under control of an inducible expression control system which also carries a selectable gene; d) introducing the vector library into a population of cell(s) expressing a transformed phenotype; e) placing the introduced transformed cell(s) from step (d) in conditions permitting expression of the cDNA and an effective concentration of an appropriate selection agent to select the cell(s) expressing the selectable gene; f) identifying the cell(s) which express the normal phenotype; and g) analyzing the cell(s) so identified so as to characterize the DNA and thus identify the tumor suppressor gene. Analogous methods to identify tumor suppressors in normal cells and to identify genes associated with unknown genetic defects are also described.

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METHOD TO IDENTIFY TUMOR SUPPRESSOR GENES

This application is a continuation-in-part of United States Application Serial No. 08/260,326, filed June 15, 1994, the contents of which are hereby incorporated by reference.

The invention disclosed herein was made with Government support under NCI/NIH Grant No. CA35675 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various references are referred to by number within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

Background of the Invention

The carcinogenic process is complex and often involves changes in the expression of two contrasting genetic elements, i.e., positive acting oncogenes and negative acting anti-oncogenes (tumor suppressor genes) (for reviews see references 1-3). Compounds displaying selective toxicity toward transformed cells overexpressing different classes of oncogenes could prove useful as potential antitumor agents and as reagents for identifying cellular targets susceptible to modification by transforming oncogenes.

Cancer is often a consequence of changes in the

expression of a number of genes. These include, dominant-acting oncogenes, tumor suppressor genes, genes affecting cell cycle and genes affecting genomic stability. In the case of tumor suppressor genes, the ability to identify and isolate these elements have proven difficult often involving extensive gene mapping and technically complex and many times unsuccessful molecular approaches. Prior to the art described in this invention, no simple and efficient way of identifying and cloning tumor suppressor genes has been available. The currently described approach is simple and effective in directly identifying potentially novel human tumor suppressor genes and directly cloning these genes. The approach, termed inducible suppression cDNA cloning, is useful in identifying both oncogene specific suppressor genes and global oncogene-independent tumor suppressor genes.

Current knowledge of tumor suppressor genes indicate that they often function as negative regulators of cell growth. Inherent in this operational definition of a tumor suppressor gene is the obvious implication that expression of a tumor suppressor gene in a target cell may evoke a loss of proliferative ability. This possibility has been demonstrated directly by reintroducing cloned tumor suppressor genes through DNA-transfection into tumor cells, i.e., growth and oncogenicity are suppressed. The growth inhibitory effect of tumor suppressor genes has prevented the previous development of functional assays permitting isolation of cells expressing novel tumor suppressor genes (anti-oncogenes).

Summary of the Invention

This invention provides a method of identifying a tumor suppressor gene of a cell(s) which comprises the following steps: a) obtaining cDNA from a normal cell(s); b) preparing a library from the cDNA of step (a), wherein the cDNA is under the control of an inducible expression control system which also carries a selectable gene; c) introducing the vector library into a population of cell(s) expressing a transformed phenotype; d) placing the introduced transformed cell(s) from step (c) in conditions permitting expression of the cDNA and an effective concentration of an appropriate selection agent to select the cell(s) expressing the selectable gene; e) identifying the cell(s) which express the normal phenotype; and f) analyzing the cell(s) so identified so as to characterize the DNA and thus identify the tumor suppressor gene.

Analogous methods to identify tumor suppressors in normal cells and to identify genes associated with unknown genetic defects are also described.

Brief Description of the Figures

Figures 1A, 1B, 1C and 1D illustrate the morphology of CREF (A), Ha-*ras*- transformed CREF (B) and Ha-*ras* plus *Krev*-1-transformed CREF clones HK B1 (C) and HK B2 (D). Monolayer cultures were fixed in formaldehyde and stained with Giemsa at approximately X 120.

Figures 2A, 2B, 2C, and 2D, illustrate the morphology of H5hr1-transformed CREF (A2) (A), a human fibroblast cDNA-induced morphological revertant H5hr1-transformed A2 CREF clone (A2/Hu-Rev/cl 5) (B), v-*src*-transformed CREF (v-*src*/cl 1) (C) and a flat v-*src* revertant CREF clone (v-*src*/A2-Hu-Rev/cl 3) (D). Monolayer cultures were fixed in formaldehyde and stained with Giemsa at approximately X 120.

Figure 3 illustrates the anchorage-independent growth of CREF and CREF cells transformed by diverse oncogenes and transformation-suppressor genes. Agar cloning efficiency (mean ± S.D.) for triplicate samples inoculated at different cell densities was determined as previously described (15). Replicate studies of agar growth varied by ≤15%.

Figures 4A, 4B, and 4C illustrate the northern analysis of steady-state mRNA in CREF and viral oncogene-transformed CREF cells. A 20-μg aliquot of total cellular RNA was run on a 1.0% agarose gel and transferred to a nylon filter. Blots were hybridized with the indicated multiprime ^{32}P-labeled gene probe. Filters were stripped and rehybridized with a multiprime ^{32}P-labeled GAPDH probe. (A) Expression of v-*raf* and GAPDH mRNA in CREF and v-*raf*-transformed CREF (v-*raf*/cl IIb). (B) Expression of v-*src* and GAPDH mRNA in CREF, v-

src-transformed CREF (v-*src*/cl 1) and flat revertant v-*src*-transformed CREF (v-*src*/A2 Hu-Rev/cl 3). (C) Expression of Ad5 E1A, Ad5 E1B and GAPDH mRNA in CREF, H5hr1-transformed CREF (A2) and flat revertant H5hr1-transformed CREF (A2/Hu-Rev/cl 5).

Figures 5A and 5B illustrate the expression of the HPV-18 and HPV-51 in CREF and HPV-18- and HPV-51-transformed CREF cells by RT-PCR. (A) Expression of HPV-18 in CREF, HPV-18-transformed CREF (HPV-18/cl T2) and HPV-51-transformed CREF (HPV-51/cl A1). (B) Expression of HPV-51 in CREF, HPV-18-transformed CREF (HPV-18/cl T2) and HPV-51-transformed CREF (HPV-51/cl A1). The specific E6 primers used for detecting HPV-18 and HPV-51 mRNA and the description of the RT-PCR procedure may be found in Materials and methods.

Figures 6A, 6B, 6C and 6D illustrate the effect of CAPE on the growth of (A) CREF, (B) v-*raf*-transformed CREF, (C) HPV-18-transformed and (D) HPV-51-transformed CREF. Cells were seeded at 2 x 10^3/3.5-cm plate, and approximately 16 hours later, the medium was changed and 0, 0.5, 1, 3, 5, 10 or 20 μg/ml CAPE added. Cell numbers from triplicate plates were determined at days 1, 2, 4, 6, 8, 10, 12 and 14. The medium was exchanged and the appropriate concentration of CAPE added every 4-5 days. Results are the average for triplicate plates which varied by ≤10%.

Figure 7A, 7B, 7C and 7D illustrate the effect of CAPE on the growth of (A) Ha-*ras*- transformed CREF (Ha-*ras*), (B) Ha-*ras* plus *Krev*-1-transformed CREF (HK B1), (C) Ha-*ras* plus *Krev*-1-transformed nude mouse tumor-derived CREF (HK B1-T) and (D) Ha-*ras* plus *Krev*-1-transformed nude mouse lung metastasis-derived CREF (HK B1-M). Experimental

details are as described in the legend to Figure 6A-6D. Cell descriptions may be found in Materials and methods.

Figures 8A, 8B, 8C and 8D illustrate the effect of CAPE on H5hr1-transformed CREF (A2), a human fibroblast cDNA-induced H5hr1-transformed revertant A2 CREF clone (A2/Hu-Rev/cl 5), v-*src*-transformed CREF (v-*src*/cl 1) and a v-*src*-transformed flat revertant CREF clone (v-*src*/A2-Hu-Rev/cl 3). Experimental details are as described in the legend to Figure 6. Cell descriptions may be found in Materials and methods.

Figure 9 illustrates the PCR amplification of the unique *Krev*-1 gene region from CREF, Ha-*ras*-transformed CREF, Ha-*ras* plus *Krev*-1-transformed CREF, Ha-*ras* plus *Krev*-1 nude mouse tumor-derived and Ha-*ras* plus *Krev*-1 metastasis-derived cells. Experimental details can be found in Materials and methods. Lane designations are as follows: M, DNA size-marker; 1, Ha-*ras* plus *Krev*-1 metastasis-derived clone, HK A3-M; 2, Ha-*ras* plus *Krev*-1 transformed clone, HK A3; 3, Ha-*ras* plus *Krev*-1 metastasis-derived clone, HK B2-M; 4, Ha-*ras* plus *Krev*-1 nude mouse tumor-derived clone, HK B2-T; 5, Ha-*ras* plus *Krev*-1 transformed clone, HK B2; 6, Ha-*ras* plus *Krev*-1 metastasis-derived clone, HK B1-M; 7, Ha-*ras* plus *Krev*-1 nude mouse tumor-derived clone, HK B1-T; 8, Ha-*ras* plus *Krev*-1 transformed clone, HK B1; 9, Ha-*ras*-transformed CREF; and 10, CREF.

Figure 10 illustrates DNA filter hybridization analysis of v-*src* in CREF, v-*src*-transformed CREF (v-*src*/cl 1) and v-*src*-transformed flat revertant CREF (v-*src*/A2-Hu-Rev/cl 3), Cellular DNA was cleaved with the restriction endonuclease PstI, 40 μg of DNA was electrophoresed on a 1% agarose gel, transferred to a nylon filter and

hybridized with a multiprime ^{32}P-labeled v-*src* probe.

Figure 11 illustrates a scheme for the inducible suppression of cDNA cloning.

Figure 12 illustrates a modified ***IscClon*** strategy.

Figure 13 illustrates a modified ***IscClon*** strategy (antisense approach).

Figure 14 illustrates a modified ***IscClon*** strategy (tetracycline responsive promoter).

Detailed Description of the Invention

This invention provides a method of identifying a tumor suppressor gene of a cell(s) which comprises the following steps: a) obtaining cDNA or mRNA from a normal cell(s); b) preparing cDNA from the cell(s) if mRNA is obtained in step (a); c) preparing a library from the said cDNA, wherein the cDNA is under the control of an inducible expression control system which also carries a selectable gene; d) introducing the vector library into a population of cell(s) expressing a transformed phenotype; e) placing the introduced transformed cell(s) from step (d) in conditions permitting expression of the cDNA and an effective concentration of an appropriate selection agent to select the cell(s) expressing the selectable gene; f) identifying the cell(s) which express the normal phenotype; and g) analyzing the cell(s) so identified so as to characterize the DNA and thus identify the tumor suppressor gene.

In this invention, cDNA from normal cells may be obtained directly from the cells. First, targeted cells may be infected with retroviral vectors which carry appropriate primer. cDNA may now be synthesized within the cell. The synthesized cDNA can therefore be obtained directly from the cell.

In an embodiment of this invention, the inducible expression control system comprises an inducible promoter. In another embodiment, the inducible expression control system comprises a repressible promoter.

An alternative method of achieving controlled expression of genes involves the use of a modified tetracycline-

repressible, bacterial tetracycline operator/repressor promoter system originally described by Gossen, M. and Bujard, H. ("Tight control of gene expression in mammalian cells by tetracycline-responsive promoters", Proc. Natl. Acad. Sci. USA, 89:5547-5551, 1992). This system uses two plasmid, pUHD15-1 and pUHD10-3. pUHD15-1 expresses a chimeric protein containing a tetracycline repressor fused to the activation domain of the herpes virus transcriptional activator, VP-16. The hybrid protein allows transactivation (tTA) of minimal promoters fused to the tetracycline operator sequences (tetO). Plasmid pUHD10-3 has a synthetic promoter with tandem repeats of a tetracycline operator (tetO) and a CMV-minimal promoter. The promoter in this plasmid is the target for activation by the tTA transcriptional activator. In the presence of tetracycline (1 μg/ml), this promoter is silent because tetracycline inhibits the ability of the tTA transactivator protein to bind to the tetO sequences. Elimination of tetracycline from the culture medium results in transcription of the gene controlled by this promoter in pUHD10-3. In this manner, cells containing the appropriate constructs can be experimentally manipulated to either express or not express normal human cDNAs by removal or addition of tetracycline. These constructs containing normal human cDNAs in an antisense orientation can be used to identify suppressor genes that when inhibited result in acquisition of a transformed phenotype by normal human cells. This approach can be applied directly to normal human cDNA libraries (either 3'-random primer cDNA or poly(dT) cDNA) (see below), cloned in a sense and antisense orientation, for use as part of the inducible suppression cDNA cloning (IScClon) strategy for identifying growth controlling and transformation suppressing tumor suppressor genes.

In one preferred embodiment of the method described above the cells expressing an transformed phenotype are CREF or CREF Trans 6 and may be transformed by either an adenovirus type 5 or the E1A region of the adenovirus type 5. In addition the cells expressing a transformed phenotype may also be transformed by an adenovirus or a retrovirus.

The transformed cells in step (c) of the method described above may be transformed by at least one oncogene or multiple oncogenes. The oncogene may be H-ras, K-ras, N-ras, v-src, v-raf, HPV-18 or HPV-51. The oncogene may be a membrane oncogene such as erb B1, erb B2, erb B3, c-fms, hst, kit, c-sis/PDGF-B and trk. Cytoplasmic oncogenes include c-abl, bcr, c-fes/fps, fyn, raf, ras and src. Other suitable oncogenes include nuclear oncogenes such as PRAD-1, erb-A, c-fos, c-jun, jun B, jum-D, mdm2-, c-myb, c-myc, B-myc, L-myc and N-myc.

A further embodiment of this invention is a method of identifying a tumor suppressor gene of a cell(s) which comprises the following steps: a) obtaining cDNA or mRNA from a normal cell(s); b) preparing cDNA from the cell(s) if mRNA is obtained in step (a); c) preparing an antisense library from the said cDNA, wherein the cDNA is under the control of an inducible expression control system which also carries a selectable gene; d) introducing the antisense library into a population of normal cell(s); e) placing the transfected normal cell(s) from step (d) in conditions permitting expression of the antisense cDNA and an effective concentration of an appropriate selection agent to select the cell(s) expressing the selectable gene; f) identifying the cell(s) which express the transformed phenotype; and g) analyzing the transformed cell(s) so identified so as to

characterize the antisense cDNA and thus identify the corresponding tumor suppressor gene.

In the methods described above the cell(s) identified in step (f) may be isolated and cultured under conditions so as to isolate and characterize the DNA and thus identify the tumor suppressor gene.

The invention provides for a method of identifying a gene in a cell(s) associated with an unknown genetic defect having a characteristic phenotype, which comprises the following steps: a) obtaining cDNA or mRNA from a normal cell(s); b) preparing cDNA from the cell if mRNA is obtained in step (a); c) preparing a library from the said cDNA, wherein the cDNA is under the control of an inducible expression control system which also carries a selectable gene; d) introducing the library into a population of cell(s) containing the unknown genetic defect having a characteristic phenotype; e) placing the cell(s) from step (d) in conditions permitting expression of the cDNA and an effective concentration of an appropriate selection agent to select the cell(s) expressing the selectable gene; f) identifying the cell(s) which express the a normal phenotype; and g) analyzing the cell(s) so identified so as to characterize the DNA and thus identify the gene associated with the unknown genetic defect. The cell(s) identified in step (f) may be isolated and cultured under conditions so as to isolate and characterize the DNA and thus identify the gene associated with the unknown genetic defect.

The gene in the cell(s) having an unknown genetic defect may be a cell(s) from a human tumor cell line or from primary human tumor isolates.

The unknown genetic defect may be associated with the following cancers, oral, esophagus, stomach, colon, rectum, liver, pancreas, larynx, lung, melanoma, skin, breast, cervix uteri, uterus, ovary, prostate, bladder, kidney, brain, non-hodgkin's lymphoma, hodbkin's disease, multiple myeloma and leukemia.

In one embodiment of the invention the inducer is removed from the cell(s) identified and isolated in step (e) prior to culturing the cell(s) in the methods described above. The inducible promoter may be Zn^{2+} metallothionein promoter, metallothionein-1 promoter, human metallothionein IIA promoter, lac promoter, lac0 promoter, mouse mammary tumor virus early promoter, mouse mammary tumor virus LTR promoter, triose dehydrogenase promoter, herpes simplex virus thymidine kinase promoter, simian virus 40 early promoter or retroviral myeloproliferative sarcoma virus promoter.

In one preferred embodiment the inducible promoter is a mouse mammary tumor early virus promoter. The promoter may be contained in a plasmid, an adenoviral vector or a retroviral vector.

The selectable gene may be neomycin phosphotransferase, hygromycin, puromycin, G418 resistance, histidinol dehydrogenase or dihydrofolate reductase gene. One preferred embodiment of the invention is a selectable gene which is a neomycin phosphotransferase gene.

The tumor suppressor gene(s) identified by the methods described above may be operatively linked to a promoter of RNA transcription. Further embodiments of the invention include: a vector which comprises the tumor suppressor gene; a virus comprising the tumor suppressor

gene; a polypeptide encoded by the tumor suppressor gene and an antibody capable of binding to the polypeptide.

Further embodiments of the invention include a non-human mammal whose germ cells or somatic cells contain a recombinant tumor suppressor gene introduced into the mammal at an embryonic stage. In additions the invention provides for a method of treating cells *ex vivo* which comprises contacting cells with the vector comprising the tumor suppressor gene so as to transform the cells and express the tumor suppressor gene discovered by the methods herein (see Leder, P. et al., U.S. Patent No. 5,175,383, Krimpenfort, P.J.A. et al., U.S. Patent No. 5,175,384, Wagner T.E. et al., U.S. Patent No. 5,175,385 and U.S. Patent No. 4,736,866).

As used herein the term identify or characterize the gene i.e. the tumor suppressor gene includes such methods of identification by fluorescence is situ hybridization, PCR, other nucleic acid probes and isolating, amplifying and sequencing such genes. Such methods are well known to those skilled in the art.

As used herein the term a gene associated with a unknown genetic defect means and includes a gene which causes a genetic defect. It includes also genes which are indicative or characteristic of a genetic defect.

The active component of the popular folk medicine propolis, caffeic acid phenethyl ester (CAPE) (5), displays increased toxicity toward cloned rat embryo fibroblast (CREF) cells transformed by adenovirus type 5 (Ad5) or the Ad5 E1A transforming gene versus untransformed CREF cells (5, 6). Employing CREF cells transformed by a cold-sensitive Ad5 E1A gene and an Ad5

E1A gene under the transcriptional control of a mouse mammary tumor virus promoter, evidence has been presented indicating that CAPE toxicity is a direct consequence of expression of the E1A-induced transformed phenotype (6). Transformation of the established rat embryo cell line, Rat 6, with the Ha-*ras* oncogene was also shown to increase the sensitivity of these cells to CAPE (6). CAPE and several additional caffeic acid esters inhibit azoxymethane-induced colonic preneoplastic lesions and ornithine decarboxylase, tyrosine protein kinase and lipoxygenase activities associated with colon carcinogenesis (7-9). In addition, CAPE exerts a dose-dependent growth suppressive effect on human colon adenocarcinoma, melanoma and glioblastoma multiforme cells (7, 10). In the human melanoma system, growth suppression was associated with the acquisition of morphological changes and the induction of cell surface antigenic changes suggesting a more differentiated phenotype (10). In contrast, at doses inducing growth suppression and cytoxicity in Ad5 E1A-transformed CREF or human tumor cells. CAPE was ineffective in altering the proliferative ability of normal human skin fibroblasts (6).

The mechanism by which CAPE induces its selective toxicity toward oncogene-transformed rodent cells and human tumor cells is not presently known. Further investigation of the phenomenon of CAPE-induced growth suppression and toxicity in oncogene-transformed rodent cells was of interest. For this invention CREF cells transformed by different classes of oncogenes have been employed. Evidence is presented indicating a direct relationship between CAPE sensitivity and transformation induced by diverse-acting oncogenes, including Ha-*ras*, HPV-18, HPV-51, v-*raf* and v-*src*. By using the *Krev*-1

tumor suppressor gene, which is 50% homologous to Ki-*ras* and blocks the transforming activity of Ha-*ras* and Ki-*ras* transformed cells at a post-transcriptional level (11-13), a direct relationship between expression of the transformed state, as opposed to the presence of the p21 Ha-*ras* oncogene-encoded protein, and CAPE sensitivity is also demonstrated. Additional studies have focused on human expression vector cDNA-library-induced revertant H5hr1- and v-*src*-transformed CREF cells that also display increased resistance to CAPE-induced toxicity versus their transformed counterparts. Taken together this indicate that the ability of CAPE to induce growth suppression and toxicity in transformed cells is a direct consequence of expression of the transformed phenotype as opposed to simply the presence of oncogene-encoded transforming proteins.

The technique of inducible suppression cDNA cloning eliminates these pitfalls and results in the isolation of tumor suppressor genes capable of suppressing the transformed and oncogenic phenotype of oncogene transformed cells. This approach that is outlined on Figure 11 is based on the use of an inducible promoter to selectively regulate expression of a tumor suppressor gene. In the example shown, the promoter is a mouse mammary tumor virus long terminal repeat sequence that is responsive to dexamethasone (DEX). The same approach can be used in conjunction with promoters responsive to other agents, i.e., Zn^{2+}-inducible metallotheionein promoter, IPTG-inducible (Lacswitch, Stratagene) promoter, etc. By growing cells in DEX, transcription of the human cDNA stably integrated into the target cell genome is induced, whereas expression is extinguished when DEX is removed from the medium. This allows the identification (+DEX) (revertant flat morphology) and isolation (-DEX) (wild-

type transformed morphology) of cells containing potential human tumor suppressor genes. By using target cells containing single or multiple oncogenes, or by multiple passage of the same cDNA tumor suppressor gene through cells containing different activated oncogenes, the currently described approach results in the identification of tumor suppressor genes with the capacity to revert specific oncogenic transforming events and/or tumor suppressor genes that can induce a global suppression of transformation (i.e., reversion of the transformed phenotype in cells containing different activated oncogenes, multiple activated oncogenes or undefined gene-induced or epigenic transformation related changes).

The current protocol should be very effective in isolating novel classes of tumor suppressor genes. Once identified, the novel tumor suppressor genes will prove valuable for numerous purposes. Including, designing gene-based strategies for reversing the oncogenic phenotype (adenoviral- or retroviral-based vectors carrying gene replacement constructs); identifying the proteins encoded by the novel tumor suppressor genes (enabling the development of potentially useful therapeutic reagents and diagnostic monoclonal antibodies); tumor screening and gene localization studies (diagnostic applications); and therapeutic intervention based on the development of rationally designed drugs capable of blocking specific biochemical pathways defective in cells displaying altered suppressor gene functions. By defining the precise genes and the encoded products involved in tumor suppression, it will also be possible to identify potentially novel and critical pathways mediating the neoplastic process. With this information in hand, it will be feasible to design

more effective therapeutic modalities to treat cancer and to develop approaches (and reagents) to directly reverse the consequences of oncogene activation and tumor progression.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

First Series of Experiments

Materials and Methods

Caffeic acid phenethyl ester

CAPE was synthesized as described by Grunberger et al. (5) and was kindly provided by Drs. T. Doyle and H. Wong, Bristol-Myers Squibb Co., Wallingford, CT.

Cell culture systems

The CREF cell line is a clonal derivative of the F2408 Fischer rat embryo fibroblast cell line (14, 15). Ha-*ras* transformed CREF cells (Ha-*ras*) were obtained following transfection of CREF cells with the Ha-*ras* (T24) oncogene and isolating a focus of cells displaying a transformed morphology (16, 17). Ha-*ras/Krev*-1 cells, containing the Ha-*ras* and *Krev*-1 gene, were obtained by cotransfecting Ha-*ras* cells with a hygromycin resistance gene (pRSV1.1) (18) and selecting cells resistant to hygromycin and displaying a reversion in morphology to that of untransformed CREF cells (19). The Ha-*ras/Krev*-1 clone HK B1 was used (19). Additionally, an HK B1 nude mouse tumor-derived clone (HK B1-M) and a lung metastasis-derived clone (HK B1-M) were analyzed for CAPE sensitivity (19). HPV-18- and HPV-51-transformed CREF cells were obtained following transfection with the cloned E6/E7 region of HPV-18 (20) and HPV-51 (21), respectively, and isolating morphologically transformed foci. CREF cells transformed by v-*raf* and v-*src* were obtained by transfecting CREF cells with the appropriate viral oncogene and isolating morphologically transformed foci (22, 23). A2 is a cold-sensitive host-range mutant, H5hr1, transformed CREF clone (24). A2 cells are

tumorigenic in both nude mice and syngeneic rats (25, 26). Morphological revertant of A2 cells, such as A2/Hu-Rev/cl 5, were obtained following transfection with a human expression vector library constructed in the pMAM-*neo* vector. The cloned cDNA inducing reversion in morphology of A2 cells was isolated and transfected into v-*src*-transformed CREF in morphologically revertant v-*src* cells, such as v-*src*/A2-Hu-Rev/cl 3. All cell lines were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum (DMEM-5) at 37°C in a 5% CO_2-95% air-humidified incubator.

Growth in monolayer culture and anchorage-independent growth assays

For monolayer growth, CREF and the various oncogene-transformed CREF cells were seeded at 2 x 10^3/3.5cm plate, and approximately 16 hours later, the medium was exchanged and 0, 0.5, 1, 3, 5, 10 or 20 μg/ml CAPE added. Cell numbers from triplicate plates were determined at days 1, 2, 4, 6, 8 10, 12 and 14. The medium was exchanged and the appropriate concentration of CAPE added every 4-5 days. For agar cloning studies, cells were seeded at 1 x 10^3 and 5 x 10^4/6-cm plate in 0.4% Noble agar on a 0.8% Noble agar base layer, both containing DMEM-5. Cultures were refed every 4 days with 0.4% Noble agar containing DMEM-5. Colonies >0.1 mm in diameter were identified with a calibrated grid under an Olympus inverted phase-contrast microscope after 21 days.

PCR analysis

To demonstrate the presence and retention of increased copies of the *Krev*-1 gene in the HK B1, HK B1-T and HK B1-M cell lines PCR analysis was employed. Cellular DNA was isolated and 40 μg was cleaved with the restriction enzyme BamHI. DNA samples were electrophoresed on a 1%

agarose gel overnight and the approximate 1.8 kb fragment was isolated from the gel. This DNA fragment was extracted with phenol:ethanol and precipitated in ethanol. A total of 5 μg of DNA was used as the PCR template. The primers employed to identify the unique *Krev*-1 regions (from nucleotide 556 and nucleotide 840) were: 5'TATTCTATTACAGCTCAGTCCACG3' (Seq. ID No. 1) and 5'AGGCTTCTTCTTTTCCACTGGTGT3' (Seq. ID No. 2). DNA samples were PCR amplified (34 cycles: 1 minute, 94°C, 1 minute, 60°C, 1 minute, 70°C) after addition of the appropriate *Krev*-1 region primers. The predicted 285 nt fragment was detected after electrophoresis in a 1% agarose gel and ethidium bromide staining.

DNA and RNA analysis

High-molecular-weight DNA was isolated from the transformed CREF cell lines as described (15). The presence of viral DNA sequences in these DNA samples was determined by DNA filter hybridization analysis as described (15, 24). Total cytoplasmic RNA was isolated from cells using the guanidinium-thiocyanate/cesium chloride method as described by Chirgwin et al. (27). Steady-state levels of the Ad5 E1A, Ad5 E1B, v-*raf* and v-*src* mRNAs were determined by Northern blot analysis of total-cytoplasmic RNA hybridized with appropriate random-primed ^{32}P-labeled probes (28). Northern blots were also probed with a ^{32}P-labeled GAPDH gene (29) to verify similar mRNA expression in the various cell types. Presence of HPV-18 and HPV-51 E6 gene expression in appropriate transformed CREF cells was determined by reverse transcription-polymerase chain reaction (RT-PCR) as described by Abdollahi et al. (30). Total cytoplasmic RNA was treated with 0.5 units DNase (Boehringer-Mannheim Biochemicals)/μg RNA in 15% glycerol- 10 mM Tris, pH 7.5 - 2.5 mM $MgCl_2$ - 0.1 mM EDTA - 80 mM KCl - 1mM $CaCl_2$ and

1 unit/ml RNasin (Promega®) at 30°C for 10 minutes. RNA was extracted with phenol-chloroform, precipitated with sodium acetate/ethanol and RNA pellets were resuspended in diethylpyrocarbonate-treated H_2O. One μg of total RNA was reverse transcribed with 200 units of murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) in 20 μl containing 1 mM deoxyribonucleotide triphosphates - 4 mM $MgCl_2$ - 10 mM Tris, pH 8.3 - 50 mM KCl- 0.001% gelatin, and 0.2 μg oligo-dT primer. Samples were diluted to 100 μl with buffer containing 0.2 mM deoxyribonucleotide triphosphates. 2 mM $MgCl_2$ 10 mM Tris, pH 8.3, 50 mM KCl and 0.001% gelatin. Fifty pmol of each primer, 1.5 units Taq DNA polymerase (Perkin-Elmer Cetus) were added and samples were covered with mineral oil, heated at 95°C for 5 minutes and subjected to 20 cycles of PCR in a Perkin-Elmer Thermal Cycler using 2 minutes denaturation at 95°C, 1 minute annealing at 55°C and 4 minutes polymerization at 72°C. After extraction with chloroform, 20 μg of products were electrophoresed, blotted onto nylon filters and hybridized with an HPV-18 E6- or HPV-51 E6-specific probe. The template primers for HPV-18 E6 were 5'CTGCGTCGTTGGAGTCTTTCC3' (Seq. ID No. 3) and 5'TTTGAGGATCCAACACGGCGA3' (Seq. ID No. 4) (20) and the template primers for HPV-51 were 5'GGGAATTCCTTCACAGTCCATCGCCGTTG3' (Seq. ID No. 5) and 5'GGGGGATCCAACACCATGTTCGAAGACAAG3' (Seq. ID No. 6) (21).

<u>CAPE induces growth suppression/toxicity in CREF cells transformed by diverse-acting oncogenes</u>

CREF cells transformed by wild-type and mutant adenovirus type 5 (Ad5) are sensitive to CAPE-induced growth suppression and toxicity (6). To determine if this effect is unique to Ad5-induced transformation of CREF cells or represents a more general phenomenon associated

with the transformed phenotype, CREF cells were transformed by a series of viral oncogenes, including v-*raf*, HPV-18, HPV-51, Ha-*ras* and v-*src*. Transformation by the various viral oncogenes resulted in morphological transformation (Figures 1A-1D and 2A-2D) and acquisition of anchorage-independence (Figure 3).

To ensure that CREF cells transformed by the different viral oncogenes express appropriate genetic information, Northern blotting was performed. CREF cells transformed by v-*raf* or v-*src* contained the appropriate viral mRNA (Figure 4A and 4B). Similarly, previous studies have indicated that CREF cells transformed by Ha-*ras* (CREF-*ras*), CREF cells transformed by Ha-*ras* and cotransfected with *Krev*-1 (HK B1) and tumor-derived HK-B1 (HK B1-T) and metastasis-derived HK-B1 (HK B1-M) cells produce Ha-*ras* mRNA and elevated levels of the *ras* oncogene-encoded product, p21 (19). In addition, the H5hr1-transformed CREF clone, A2, reverted to a more contact-inhibited morphology (Figures 2A-2D) following a transfection with a human fibroblast expression vector cDNA library (A2/Hu-Rev/cl 5) continued to produce both Ad5 E1A and E1B mRNAs (Figure 4C). Expression of the transformation related E6 mRNA in HPV-18- and HPV-51-transformed CREF cells was demonstrated by RT-PCR (Figures 5A and 5B). These observations indicate that the various cell lines used were transformed by the specific viral oncogene used and they express appropriate viral oncogene-encoded genetic information.

The effect of CAPE on the growth of CREF and CREF cells transformed by v-*raf*, HPV-18 and HPV-51 is shown in Figures 6A-6D. In contrast to CREF cells, which display increases in cell number even when exposed to 15 μg/ml of CAPE, 10 μg/ml of CAPE is cytostatic toward v-*raf*/cl IIb

and HPV-18/c1 T2 cells and 15 μg/ml of CAPE is cytostatic toward HPV-51/c1 A1 cells. Twenty μg/ml of CAPE is cytotoxic toward HPV-18/c1 T2 and HPV-51/c1 A1 cells. CREF cells transformed by Ha-*ras* or v-*src* are even more sensitive to the cytostatic and cytotoxic effects of CAPE. A dose of 3 μg/ml of CAPE is cytostatic toward Ha-*ras* and v-*src* cells, whereas 5 μg/ml or higher doses of CAPE are cytotoxic toward Ha-*ras*- and v-*src*-transformed CREF cells (Figures 7A-7D and Figure 8). Similar patterns of CAPE sensitivity have also been observed using additional independently derived Ha-*ras*- and v-*src*-transformed CREF clones. These results indicate that CREF cells transformed by diverse acting oncogenes become sensitive to CAPE-induced growth suppression and cytoxicity. A direct correlation between the degree of CAPE sensitivity and expression of the transformed phenotype is also indicated, i.e., transformed cells displaying enhanced growth in agar are more sensitive to CAPE than cells displaying a lower efficiency of agar growth.

<u>CAPE-induced growth suppression/toxicity correlates directly with expression of the transformed phenotype in Ha-*ras*-transformed CREF cells</u>

The studies described above indicate that acquisition of the transformed phenotype by CREF cells, irrespective of the transforming viral oncogene used, results in an increase in sensitivity to CAPE-induced growth suppression. To determine if reversion of the transformed phenotype results in a change in sensitivity to CAPE, Ha-*ras* and Ha-*ras*/*Krev*-1 expressing CREF cells (19) have been used (Figures 1B, 1C, 7A and 7B). CREF cells transformed by Ha-*ras*, Ha-*ras*/cl 5, are morphologically transformed, grow with approximately a 38% efficiency in agar and they induce both tumors and

metastases in nude mice and syngeneic rats (Figures 1 and 3) (19). In contrast, CREF cells transformed by both Ha-*ras* and *Krev*-1 display a reversion in morphology to a more normal CREF-life phenotype, a reduction in anchorage independence and a suppression in tumorigenic and metastatic potential (19) (Figures 1 and 3). HK B1 cells do, however, induce both tumors (HK B1-T) and lung metastases (HK B1-M) in nude mice after a long latency period (19). Unlike HK B1 parental cells, which display a similar pattern of gene expression as untransformed CREF cells, HK B1-T and specifically HK B1-M cells display a reversion in their gene expression to that of Ha-*ras* cells (19). HK B1-T and HK B1-M cells retain the original *Krev*-1 gene as indicated by PCR analysis (Figure 9), continue to synthesize *Krev*-1 mRNA (19) and Ha-*ras* mRNA (19) and like HK B1 cells continue to synthesize the Ha-*ras*-encoded p21 protein (19). These results indicate that the *Krev*-1 gene can modify expression of the transformed state in Ha-*ras* cells at a post-transcriptional level. In this respect, this model is ideal for determining if CAPE-induced changes are related simply to the presence of the oncogene-encoded products or to the actual status of expression of the transformed phenotype.

As shown in Figure 7A, Ha-*ras*/cl 5 cells are sensitive to both CAPE-induced growth suppression and cytotoxicity. In contrast, HK B1 cells, which display a CREF-like phenotype, acquire resistance to CAPE. In fact, even the highest dose of CAPE tested, 20 μg/ml, does not result in a loss of proliferative ability in HK B1 cells. A similar reversion to CAPE resistance is observed in two additional independent Ha-*ras*/*Krev*-1 clones, HK B2 and HK A3 (19). Escape from transformation-suppression following tumor- and metastasis-induction in nude mice

results in HK B1-derived cells that have reacquired sensitivity to CAPE-induced growth suppression and cytotoxicity (Figure 7B). HK B1-T and HK B1-M cells exhibit an increase in anchorage independence in comparison with HK B1 parental cells (Figure 3) (19). HK B1-T cells also display an increase in anchorage-independence and they are more sensitive to CAPE than HK B1-M cells (Figures 3 and 7D). These results indicate that sensitivity to CAPE correlates directly with expression of the transformed state in CREF cells, as opposed to the mere presence of oncogene-encoded gene products.

CAPE-induced growth suppression/toxicity correlates directly with expression of the transformed phenotype in H5hr1- and v-*src*-transformed CREF cells

The studies described above using CREF cells transformed by Ha-*ras* and Ha-*ras* plus *Krev*-1 indicate a direct correlation between the transformed phenotype and CAPE sensitivity. For further investigation of this relationship, two transformation-revertant systems have been used. Using a modification of the strategy described by Kitayama et al. (11) which has resulted in the identification and isolation of the *Krev*-1 suppressor gene, an H5hr1-transformed CREF clone, A2, was transfected with an expression vector library containing cDNAs from normal human skin fibroblasts cloned into the pMAM*neo* vector. Cells were then selected in medium containing G418 and 10^{-7} M dexamethasone. A series of G418-resistant colonies, displaying a flat CREF-like morphology, was isolated and expanded for analysis. The A2 revertant clone, A2/Hu-Rev/cl 5 were used. This revertant clone displays a CREF-like morphology, has an extended population doubling-time, grows with reduced efficiency in agar and has an increased latency time for

tumor formation in nude mice (Figures 2 and 3). In contrast to these altered biological properties, both A2 and A2/Hu-Rev/cl 5 cells express similar levels of Ad5 E1A and E1B mRNA (Figure 4C). A2 cells are extremely sensitive to CAPE with 1.5 μg/ml resulting in growth suppression and 3 μg/ml or higher levels of CAPE inducing a cytotoxic effect (6) (Figure 8). In contrast, although 5, 10 and 20 μg/ml of CAPE was cytostatic toward A2/Hu-Rev/cl 5 cells, no cytotoxic effect was apparent in the revertant cells.

The cDNA inducing reversion of the transformed phenotype in A2 cells was isolated and transfected into v-*src*-transformed CREF cells. Using a similar protocol as used in A2 transfections, a series of G418-resistant v-*src*-transformed CREF colonies displaying a reverted CREF-like morphology were isolated and expanded for further analysis (Figure 2). The v-*src*-flat revertant, v-*src*/A2-Hu-Rev/cl 3 were used. In contrast to A2/Hu-Rev/cl 5 cells that continue to express Ad5 gene products, v-*src*/A2-Hu-Rev/cl 3 cells do not synthesize v-*src* mRNA that is detectable by Northern blotting (Figure 4B). Southern blotting indicates that the v-*src* gene is still present in v-*src*/A2-Hu-Rev/cl 3 cells (Figure 10). As observed with A2/Hu-Rev/cl 5 versus A2 cells, v-*src*/A2-Hu-Rev/cl 3 cells display increased resistance to CAPE as compared to parental v-*src* cells (Figure 8). These results provide compelling evidence indicating a direct correlation between expression of the transformed phenotype and CAPE sensitivity.

The ability of diverse acting cellular and viral oncogenes to induce transformation in primary and established rodent fibroblast cells indicates that transformation can proceed by different mechanisms (31-

33). Irrespective of the transforming agent, transformed fibroblast cells often express similar cellular and biochemical changes (31, 32). Modifications i cellular phenotype associated with transformation of rodent fibroblasts often include decreased population doubling times, increased saturation densities, acquisition of anchorage-independence and tumorigenesis in athymic nude mice (31, 32). Compounds that display enhanced cytostatic/cytotoxic effects toward transformed versus normal cells represent potentially important agents for cancer therapy (34). It is demonstrated that CAPE displays a selective antiproliferative effect toward CREF cells transformed by viral oncogenes that display different modes of action, including Ad5, v-*raf*, v-*src*, Ha-*ras*, HPV-18 and HPV-51. Evidence is also provided that both expression of the transformed phenotype and the degree of expression of transformation (measured by anchorage-independence) as opposed to simply the presence of the transforming oncogene product, are the mediators of CAPE sensitivity.

Previous studies have shown the effect of CAPE on growth and DNA synthesis in CREF cells infected, transfected or stably transformed by wild-type or mutant Ad5-transforming genes (6) has been analyzed in detail. CAPE inhibited, in a dose-dependent manner, both *de novo* and carcinogen-enhanced transformation of CREF cells by H5hr1. When transfected into CREF cells, a cold-sensitive Ad5 E1A gene only resulted in an inhibition in colony formation by CAPE when cells were grown at a permissive temperature for expression of the transformed phenotype, i.e., 37°C. CAPE was also most effective in inhibiting DNA synthesis in CREF cells containing either a wild-type Ad5 E1A gene (at 32 or 37°C) or a cold-sensitive Ad5 E1A gene inducing a transformed phenotype

(at 37°C). A direct-requirement for a functional Ad5 E1A gene, capable of eliciting the transformed state, and CAPE sensitivity was demonstrated by using CREF cell stably transformed by a cold-sensitive A5 E1A gene or an Ad5 E1A gene under the transcriptional control of a mouse mammary tumor virus promoter. To determine the effect of the individual transforming proteins of the Ad5 A1A gene on CAPE sensitivity, CREF cells were stably transformed with cDNAs encoding either the 13S or the 12S E1A mRNA, which produce the 289 and 243 amino acid Ad5 transforming proteins respectively (6). Using these cell lines, it was demonstrated that CAPE was more growth suppressive toward cells expressing both transforming proteins followed by CREF cells transformed with the 13S cDNA and least effective against cells expressing only the 12S cDNA (6). It is demonstrated that CAPE-induced toxicity is eliminated when the H5hr1-transformed CREF clone, A2, is reverted to a more normal CREF-like phenotype by expression of a transfected human fibroblast cDNA. Revertant A2 clone, such as A2/Hu-Rev/cl 5, continue to express both the A5 E1A and E1B transforming genes. These results provide evidence that CAPE sensitivity in Ad5-transformed CREF cells is directly dependent on expression of the transformed state, as opposed to simply the presence of Ad5 E1A-transforming gene in A2/Hu-Rev/cl 5 cells.

The conclusion that CAPE-induced toxicity is a consequence of the extent of expression of the transformed phenotype is further supported by the following. Revertant of v-*src* transformed CREF cells, containing a human cDNA suppressor gene identified in A2/Hu-Rev/cl 5 cells, display a stable reversion in transformation-related properties and reacquire an increased resistance to CAPE-induced growth suppression

and toxicity. The v-*src* revertant clone, v-*src*/A2-Hu-Rev/cl 3, no longer expresses v-*src* mRNA, indicating that the increased resistance to CAPE may be mediated by the absence of the transforming oncogene products. In the case of *Krev*-1-induced revertant of Ha-*ras*-transformed CREF cells, acquisition of a reverted transformation phenotype is not associated with changes in the levels of the Ha-*ras* oncogene products (19). Ha-*ras* cells are sensitive to CAPE-induced growth suppression and toxicity, whereas *Krev*-1 revertant Ha-*ras* transformed CREF clones, such as HK B1, are resistant to the cytostatic and cytotoxic effects of CAPE. When HK B1 cells escape transformation suppression, following long latency times in nude mice, tumors (HK B1-T) and lung metastases (HK B1-M) develop. The tumor- and lung metastasis-derived HK B1 clones coordinately display transformation-related properties and CAPE sensitivity. In addition, HK B1-T cells display a greater *in vitro* expression of anchorage-independence than the HK B1-M clones, and these cells are more sensitive to CAPE. These studies indicate a direct relationship between expression of the transformed state, with and without retention of the oncogene-encoded genetic information, and sensitivity to CAPE.

The mechanism by which CAPE induces its cytostatic and cytotoxic activity toward CREF cells transformed by diverse acting oncogenes is not presently known. In the case of Ad5 and human papilloma virus-induced transformation, common cellular genes may provide targets for oncoprotein interactions (35, 36). These include, an interaction between the Ad E1A- and HPV E7-encoded gene products and specific cellular proteins, such as the retinoblastoma gene product (p105-RB) and the p105-RB related proteins cyclin A (p107) and p130 (37).

Similarly, the Ad5 E1B- and HPV E6-encoded gene products specifically target the p53 tumor suppressor protein for inactivation (38). These observations have led to the hypothesis that induction of transformation by DNA viruses such as Ad and HPV may involve a direct inactivation of cellular gene products that normally function as suppressors of the transformed and oncogenic phenotype. In contrast, current evidence suggests that Ha-*ras* induced transformation is a consequence of the intrinsic guanosine triphosphatase activity of the oncogenic *ras*-encoded protein, p21, which functions as a component of the guanine nucleotide-binding protein signal transduction pathway in cells (39, 40). Similarly, the v-*src* gene encodes a membrane-associated tyrosine-specific kinase that is involved in cell signaling pathways and which may also involve small guanine nucleotide-binding proteins as target molecules (41). The transforming gene of murine sarcoma virus 3611, v-*raf* encodes a cytosolic serine/threonine kinase (22). The cellular homologue of v-*raf*, c-*raf*-1, is involved in regulating early gene expression changes associated with growth-factor stimulation of cells and acts downstream of *ras* (22, 42). Since all of the viral oncogenes described above render CREF cells sensitive to the antiproliferative effects of CAPE, apparently multiple and alternative biochemical changes that ultimately culminate in expression of the transformed state determine CAPE susceptibility. Furthermore, as indicated above the relative degree of CAPE-sensitivity is also directly related to expression of the transformed state, i.e., cells displaying greater anchorage-independence are more sensitive to CAPE-induced growth suppression and toxicity. Additional studies are necessary to define the common down-stream target that is shared by all of these oncogenes and which serves as the

mediator of CAPE sensitivity. As a first step, it will be necessary to define the site within a cell in which CAPE initially interacts, i.e., the cell membrane, internal organelles, such as the mitochondria or the nucleus, specific enzymes, etc. Preliminary studies using [^{3}H]-labeled CAPE indicate no differential binding between CAPE-sensitive cells, such as A2, versus CAPE-resistant cells, such as CREF. These observations suggest that an intracellular target may prove to be the site of interaction with CAPE.

Elucidation of the biochemical changes that render a cell sensitive to CAPE-induced antiproliferative and cytotoxic activities could result in the identification of common cellular processes altered during oncogenic transformation. This information would prove beneficial in the rational design of chemotherapeutic agents that display antitumor activity toward cancer cells by exploiting common transformation endpoints as targets. In this context, appropriately designed agents would display selective activity toward neoplastic cells that developed as a consequence of the effects of diverse-acting oncogenes and/or the inactivation of diverse-acting tumor suppressor genes.

Second Series of Experiments

Inducible suppression cDNA cloning (***IScClon***) is a procedure for identifying and cloning growth inhibitory tumor suppressor genes. This approach can also be used to define genes controlling cellular differentiation and cell growth potential (senescence). ***IScClon*** is based on a hypothesis that constitutive expression of specific tumor suppressor genes in transformed and tumorigenic cell lines can result in a reversion in phenotype to a ore normal cellular state. In many contexts, this transformation-reversion may correlate with an irreversible loss in proliferative capacity, thereby, preventing the isolation of cells expressing novel tumor suppressor genes, ***IScClon*** permits the identification of cells containing tumor suppressor genes (based on a reversion in cellular morphology under conditions that induce tumor suppressor function) and allows growth of tumor suppressor gene containing cells (based on a return to a transformed state and/or resumption of growth under conditions that prevent tumor suppressor function). The ***IScClon*** approach should prove amenable to identifying and cloning genes mediating reversion of any transforming event, induced by either known or unidentified genetic changes.

Recent studies have focused on the application of ***IScClon*** for reverting the transformed phenotype of CREF cells transformed by human papilloma virus type 18 (HPV-18) (J. Lin, Z.-z, Su, D. Grunberger, S.G. Zimmer & P.B. Fiher, "Expression of sensitivity to growth phenotype induced by diverse acting viral oncogenes mediates sensitivity to growth suppressio induced by caffeic acid phenethyl ester (CAPE)", Int. J. Oncology, 5:5-15, 1994). CREF HPV-18/cl T2 cells were transfected with a human fibroblast random

3'-primer or poly (dT) cDNA expression library cloned into a pMAM-neo vector (allowing inducible expression in the expression in the presence of dexamethasone (DEX)). When grown in the presence of G418, neomycin resistant-transformed CREF HPV-18/cl T2 colonies developed. Application of DEX for 48 hours permitted the identification of colonies containing cells with a CREF-life morphology. Removal of DEX and further growth resulted in specific colonies that degenerated, remained morphologically normal or reverted back to the transformed phenotype. Both morphologically normal (constitutive in the absence of DEX) and inducible reverted (normal in the presence of DEX and transformed in the absence of DEX) colonies have been isolated and are being characterized.

Additional studies have been performed using the cold-sensitive host-range type 5 adenovirus mutant, H5hr1, transformed CREF clone, A2. A2 cells were transfected with a human fibroblast random 3'-primer or poly (dT) cDNA expression library cloned into a pMAM-neo vector (allowing inducible expression in the presence of dexamethasone (DEX)). Growth in the presence of G418 results in neomycin resistant-transformed A2 colonies. Addition of DEX for 48 hours permitted the identification of A2 colonies reverting to a more normal CREF-like morphology. As with HPV-18-transformed CREF cells, removal of DEX and further growth resulted in specific colonies that degenerated, remained morphologically normal or reverted back to the transformed phenotype. Both morphologically normal A2 (constitutive in the absence of DEX) and inducible reverted A2 (normal in the presence of DEX and transformed in the absence of DEX) colonies have been isolated and are being characterized.

The studies briefly described above suggest that both DEX-inducible and DEX-constitutive morphological revertant transformed cells will prove valuable in identifying genetic elements with the capacity to revert the transformed and oncogenic capacity of tumor cells. The modified ***IScClon*** strategy is shown in Figure 12. The modified ***IScClon*** using antisense cDNAs shown in Figure 13. The modified ***IScClon*** using tetracycline responsive promoters is shown in Figure 14.

Construction of Random Unidirectional Linker-Primer, 3'Random Primer cDNA and Poly(dT) cDNA, Libraries from Normal Human Skin Fibroblast Cells

For the inducible suppression cDNA cloning (IScClon) approach, both 3'-random primer and poly(dT) primer cDNA libraries are constructed from normal human skin fibroblast mRNA. The procedures are as described by Stratagene® and in detail in P.G., Reddy, Z.-z. Su and P.B. Fisher, "Identification and cloning of genes involved in progression of transformed phenotype", Gene and Chromosome Analysis, K.W. Adolph (Ed), Methods In Molecular Genetics, Vol. 1, Academic Press, San Diego, CA, pp 68-102, 1993; and H. Jiang and P.B. Fisher, "Use of a sensitive and efficient subtraction hybridization protocol for the identification of genes differentially regulated during the induction of differentiation in human melanoma cells", Mol. Cell. Different., 1(3):P285-299, 1993.

Total cellular RNA from normal human skin fibroblasts is isolated by the guanidinium isothiocyanate/CsCl centrifugation procedure and poly (A+) RNA was selected following oligo(dT) cellulose chromatography. A total of 10 μg of mRNA and 5 μg of random-primer (Stratagene, Cat.

901151, 6/2/94) starts reverse transcription (3'-random primer cDNA library construction). Alternatively, a total of 5 μg of mRNA and 2.5 μg of a oligo(dT) primer (supplied in the Stratagene, ZAP-cDNA synthesis kit) starts reverse transcription (poly(dT) primer cDNA library construction). Protocols for constructing cDNA libraries are described by Stratagene and Reddy et al (1993) and Jiang and Fisher (1993).

Cloning of the 3'-random primer and poly(dT) primer cDNA libraries into an inducible expression vector (e.g. pMAMneo vector

Double-stranded phagemid DNAs (from λ ZAP) are prepared from the 3'-random primer and poly(dT) human fibroblast (HF) cDNA libraries using the mass excision procedure (Stratagene®) as described by Jiang and Fisher (1993). Briefly, 1 x 10^7 pfu of phagemids containing HF cDNA library are mixed with 2 x 10^8 SOLR strain of Escherichia coli and 2 x 10^8 pfu of ExAssist helper phage in 10 mM $MgSO_4$, followed by adsorption at 37°C for 15 minutes. After the addition of 10 ml LB medium, the phagemid/bacteria mixture is incubated with shaking at 37°C for 2 hours, followed by incubation at 70°C for 15 minutes to heat inactivate the bacteria and the λ ZAP phage particles. After centrifugation at 4000 g for 15 minutes, the supernatant is transferred to a sterile polystyrene tube and stored at 4°C before use.

To produce double-stranded DNA, 5 x 10^7 pfu of the phagemids is combined with 1 x 10^9 SOLR strain of Escherichia coli, which are nonpermissive for the growth of helper phage and therefore prevent coinfection by helper phage (Ref. 22 from Jiang and Fisher, 1993), in 10 mM $MgSO_4$, followed by adsorption at 37°C for 15 minutes.

The phagemids/bacteria are transferred to 250 ml of LB medium containing 50 μg/ml ampicillin and incubated with shaking at 37°C overnight. The bacteria are isolated by the alkali-lysis method (Ref. 18 from Jiang & Fisher, 1993) and purified through a QIAGEN-tip 500 column (QIAGEN Inc., Chatsworth, CA).

The purified double-stranded cDNA-containing phagemid is digested with the restriction endonucleases EcoRI and XhoI. The liberated cDNA inserts are isolated from the vector following digesting by electrophoresis in 1% agarose and electroelution. The purified cDNA inserts are ligated into the pMAMneo (Clontech) vector that has been incubated with the same restriction enzymes (EcoRI and XhoI) (inserts: vector = 4:1). The ligated complex is then transfected into XL-1 blue strain of Escherichia coli resulting in the production of a dexamethasone (DEX)-inducible human fibroblast cDNA library. The production and purification of human fibroblast cDNA inserts/pMAMneo DNA is by the procedure developed by QIAGEN (QIAGEN plasmid Maxi protocol). The human fibroblast expression cDNA library (50μl) in 500 ml LB medium with 50 μg/ml ampicillin overnight with shaking at 37°C. Plasmid DNA is isolated by the alkali-lysis method (Ref 18 from Jiang and Fisher, 1993) and purified through a QIAGEN-tip 500 column.

Identification of Cells Containing Growth and Tumor Suppressor Genes Using the Inducible Suppression cDNA Cloning (IScClon) Approach.

The IScClon approach is shown in Fig. 12. Approximately 1 X 10^6 target mammalian cells (CREF or CREF-Trans 6 cells containing transfected oncogenes (including, but not limited to Ad5, Ad5 mutant, v-src, HPV-18, Ha-ras) or

high molecular weight (HMW) human tumor DNA; or human tumor cells) are transfected with 10 μg of plasmid HF cDNA library/pMAMneo DNA (HF cDNA library cloned in a sense orientation for transfer into CREF, CREF-Trans 6 or human tumor cells) by the calcium phosphate, lipofectin or electroporation technique. Transfected cells are replated 48 hr later and after 24 hr 300 μg/ml of G418 is added. After colonies form, approximately 7 to 21 days depending on the cell type, 10^{-6} M DEX is added. After 24 to 48 hr, plates are scanned microscopically and colonies displaying a morphologically reverted phenotype (to a normal morphology with sense inducible cDNAs) are identified and circled. DEX is then removed, colonies are isolated with metal cloning cylinders and maintained for further analysis as independent cell strains. These cell strains containing putative human growth suppressing and tumor suppressor genes can then be used to clone, sequence and characterize the potentially novel human tumor growth and transformation related tumor suppressor genes. At present, two types of cell strains have been identified: (a) cell clones that are reverted to a normal phenotype by treatment with DEX, but retain a normal cellular phenotype even in the absence of DEX; and (b) cell clones that display a reversible phenotype in the presence (normal phenotype) and absence (transformed phenotype) of DEX (see Figure 12).

The IScClon approach using antisense cDNA constructs is shown in Figure 13. Approximately 1 x 10^6 target normal human cells (skin fibroblast, epithelial, melanocyte, astrocyte, keratinocyte or other normal human cell type) are transfected with 10 μg of plasmid HF cDNA library/mMAMneo DNA (HF cDNA library cloned in an antisense orientation) by the calcium phosphate, lipofectin or electroporation technique. Transfected

cells are replated 48 hours later and after an additional 24 hours 300 to 500 μg/ml of G418 is added. Depending on the normal human cell type used transfected cells may be plated on feeder-layers consisting of irradiated CREF cells to improve colony forming efficiency. After colonies form, approximately 14 to 28 days depending on the cell type, 10^{-6}M DEX is added. After 24 to 48 hours, plates are scanned microscopically and colonies displaying a morphologically reverted phenotype (to a transformed state) are identified and circled. Alternatively, colonies displaying growth under conditions limiting growth of the normal cell type, i.e., removal of specific growth factors, are also identified. DEX is then removed, colonies are isolated with metal cloning cylinders and maintained for further analysis as independent cell strains. Changes indicating potentially interesting antisense cDNAs include: changes in cellular morphology and growth properties (morphological transformation, anchorage-independence, acquisition of tumorigenic potential), ability to grow in the absence of specific growth factors (insulin, platelet derived growth factor TPA), loss of lineage-specific differentiation markers (melanin production, enzymatic changes, absence of cell surface antigenic markers) and unlimited growth potential (immortality and the loss of senescence). These cell strains containing putative human growth suppressing, tumor suppressing, differentiation suppressing and/or senescence suppressing genes can then be used to clone, sequence and characterize the potentially novel suppressor gene (see Figure 12).

the IScClon approach using tetracycline (TET) suppressible tetracycline-responsive promoters is shown in Figure 14. Approximately 1 X 10^6 target normal mammalian cells (CREF or CREf-Trans 6 cells containing

transfected oncogenes (including, but not limited to Ad5, Ad5 mutant, v-src, HPV-18, Ha-ras) or high molecular weight (HMW) human tumor DNA; or human tumor cells are transfected with 10μg of plasmid PUHD15-1 and 1 μg of pSV2-neo DNA by the calcium phosphate, lipofectin or electroporation technique. Transfected cells are plated 48 hours later and after an additional 24 hr 300 to 500 μg/ml of G418 is added. G418 resistant colonies are identified after 7 to 21 days, isolated and cell strains expressing the tetracycline repressor fused to the activation domain of the herpes virus transcriptional activator, VP-16, are identified. These transformed cells are then transfected with 10 μg of plasmid pUHD10-3 containing the HF cDNA library and 1 μg of pRSV1.1 DNA (containing a hygromycin resistance gene) by the calcium phosphate, lipofectin or electroporation technique. Transfected cells (grown in the presence of 1 μg/ml of tetracycline) are replated 48 hr later and after an additional 24 hr 100 to 400 μg/ml of hygromycin plus 1 μg/ml tetracycline is added. Hygromycin resistant colonies are identified 7 to 21 days later. Tetracycline is removed and after 48 hr, plates are scanned microscopically and colonies displaying a morphologically reverted normal cellular phenotype are identified and circled. Tetracycline (1 μg/ml) is then added, colonies are isolated with metal cloning cylinders and maintained for further analysis as independent cell strains. These cell strains containing putative human growth suppressing and tumor suppressor genes can then be used to clone, sequence and characterize the potentially novel human tumor growth and transformation related tumor suppressor gene (see Figure 14).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Fisher, Paul B.

(ii) TITLE OF INVENTION: METHOD TO IDENTIFY TUMOR SUPPRESSOR GENES

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Cooper & Dunham, LLP
(B) STREET: 1185 Avenue of the Americas
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: U.S.A.
(F) ZIP: 10036

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: White, John P.
(B) REGISTRATION NUMBER: 28,678
(C) REFERENCE/DOCKET NUMBER: 0575/45571-A-PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (212) 278-0400
(B) TELEFAX: (212) 391-0525

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(ii) MOLECULE TYPE: DNA (genomic)

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What is claimed is:

1. A method of identifying a tumor suppressor gene of a cell(s) which comprises:
   a) obtaining cDNA or mRNA from a normal cell(s);
   b) preparing cDNA from the cell(s) if mRNA is obtained in step (a);
   c) preparing a library from the said cDNA, wherein the cDNA is under the control of an inducible expression control system which also carries a selectable gene;
   d) introducing the library into a population of cell(s) expressing a transformed phenotype;
   e) placing the introduced transformed cell(s) from step (d) in conditions permitting expression of the cDNA and an effective concentration of an appropriate selection agent to select the cell(s) expressing the selectable gene;
   f) identifying the cell(s) which express the normal phenotype; and
   g) analyzing the cell(s) so identified so as to characterize the DNA and thus identify the tumor suppressor gene.

2. The method of claim 1, wherein the inducible expression control system comprises an inducible promoter.

3. The method of claim 1, wherein the inducible expression control system comprises a repressible promoter.

4. The method of claim 1, wherein the cell(s) identified in step (f) are isolated and cultured under conditions so as to isolate and characterize

the DNA and thus identify the tumor suppressor gene.

5. The method of claim 1, wherein the cells expressing an transformed phenotype are CREF or CREF Trans 6.

6. The method of claim 5, wherein the CREF cells or CREF Trans 6 cells are transformed by either an adenovirus type 5 or the E1A region of the adenovirus type 5.

7. The method of claim 1, wherein the cells expressing a transformed phenotype are transformed by an adenovirus or a retrovirus.

8. The method of claim 4, wherein the inducer is removed from the cell(s) identified and isolated in step (f) prior to culturing the cell(s).

9. The method of claim 2, wherein the inducible promoter is Zn^{2+} metallothionein promoter, metallothionein-1 promoter, human metallothionein IIA promoter, lac promoter, lacO promoter, mouse mammary tumor virus early promoter, mouse mammary tumor virus LTR promoter, triose dehydrogenase promoter, herpes simplex virus thymidine kinase promoter, simian virus 40 early promoter or retroviral myeloproliferative sarcoma virus promoter.

10. The method of claim 9, wherein the inducible promoter is a mouse mammary tumor virus early promoter.

11. The method of claim 1, wherein the selectable gene is neomycin phosphotransferase, hygromycin,

puromycin, G418 resistance, histidinol dehydrogenase or dihydrofolate reductase gene.

12. The method of claim 11, wherein the selectable gene is a neomycin phosphtransferase gene.

13. The method of claim 1, wherein the transformed cells in step (d) are transformed by at least one oncogene.

14. The method of claim 13, wherein the oncogene is H-ras, K-ras, N-ras, v-src, v-raf, HPV-18 or HPV-51.

15. The method of claim 1, wherein the transformed cells in step (d) are transformed by multiple oncogenes.

16. The tumor suppressor gene identified by the method of claim 1.

17. A method of identifying a tumor suppressor gene of a cell(s) which comprises:

a) obtaining cDNA or mRNA from a normal cell(s);

b) preparing cDNA from the cell(s) if mRNA is obtained in step (a)

c) preparing an antisense library from the said cDNA, wherein the cDNA is under the control of an inducible expression control system which also carries a selectable gene;

d) introducing the antisense library into a population of normal cell(s);

e) placing the transfected normal cell(s) from step (d) in condition permitting expression of the antisense cDNA and an effective concentration of an appropriate selection agent to select the cell(s) expressing the selectable

gene;

f) identifying the cell(s) which express the transformed phenotype; and

g) analyzing the transformed cell(s) so identified so as to characterize the antisense cDNA and thus identify the corresponding tumor suppressor gene.

18. The method of claim 17, wherein the cell(s) identified in step (f) are cultured under conditions so as to isolate and characterize the DNA and thus identify the tumor suppressor gene.

19. The tumor suppressor gene of claim 16 operatively linked to a promoter of RNA transcription.

20. A vector which comprises the tumor suppressor gene of claim 16.

21. A virus comprising the tumor suppressor gene of claim 20.

22. A polypeptide encoded by the tumor suppressor gene of claim 16.

23. An antibody capable of binding to the polypeptide of claim 22.

24. A non-human mammal whose germ cells or somatic cells contain a recombinant tumor suppressor gene of claim 16, introduced into the mammal at an embryonic stage.

25. A method of treating cells *ex vivo* which comprises contacting cells with the vector of claim 20 so as

to transform the cells and express the tumor suppressor gene.

26. A method of identifying a gene in a cell(s) associated with an unknown genetic defect having a characteristic phenotype, which comprises:

a) obtaining cDNA or mRNA from a normal cell(s);

b) preparing cDNA from cell(s) if mRNA is obtained in step (a);

c) preparing a library from the said cDNA, wherein the cDNA is under the control of an inducible expression control system which also carries a selectable gene;

d) introducing the library into a population of cell(s) containing the unknown genetic defect having a characteristic phenotype;

e) placing the cell(s) from step (d) in conditions permitting expression of the cDNA and an effective concentration of an appropriate selection agent to select the cell(s) expressing the selectable gene;

f) identifying the cell(s) which express a normal phenotype; and

g) analyzing the cell(s) so identified so as to characterize the DNA and thus identify the gene associated with the unknown genetic defect.

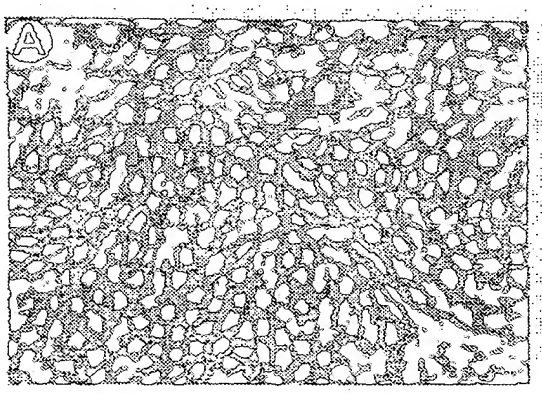
27. The method of claim 26, wherein the cell(s) identified in step (f) are isolated and cultured under conditions so as to isolate and characterize the DNA and thus identify the gene associated with the unknown genetic defect.

28. The method of claim 26, wherein the gene in the cell(s) having an unknown genetic defect is a

cell(s) from a human tumor cell line.

29. The method of claim 26, wherein the gene in the cell(s) having an unknown genetic defect is a cell(s) from primary human tumor isolates.

FIGURE 1A



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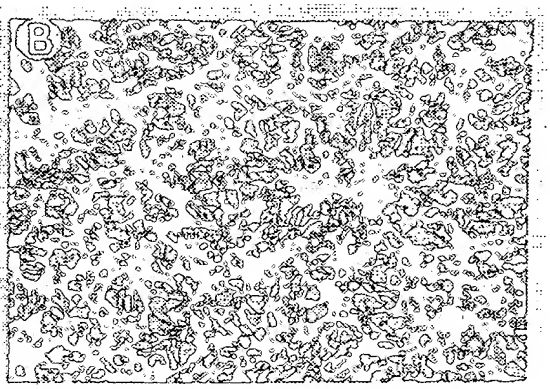
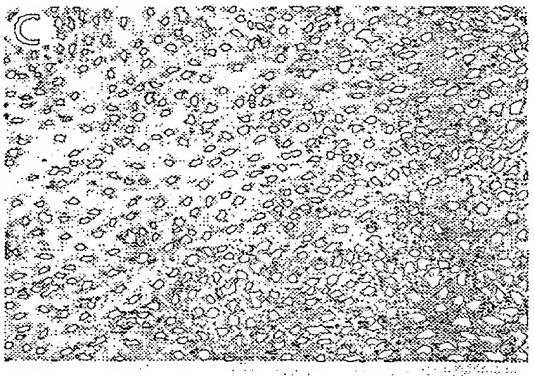


FIGURE 1C



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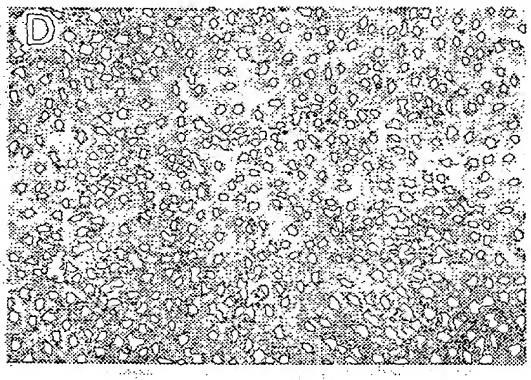

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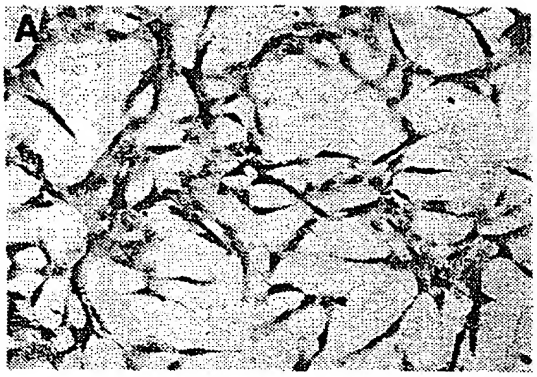

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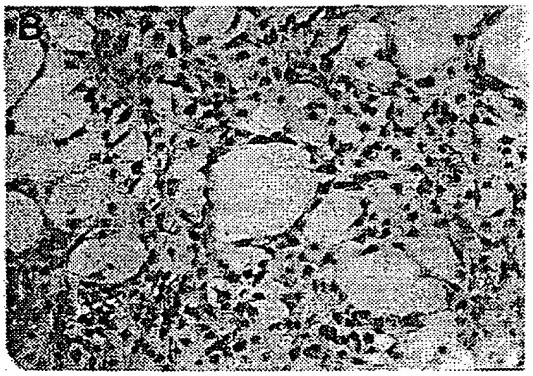

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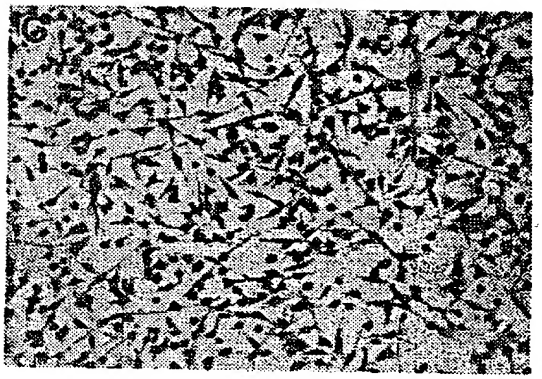

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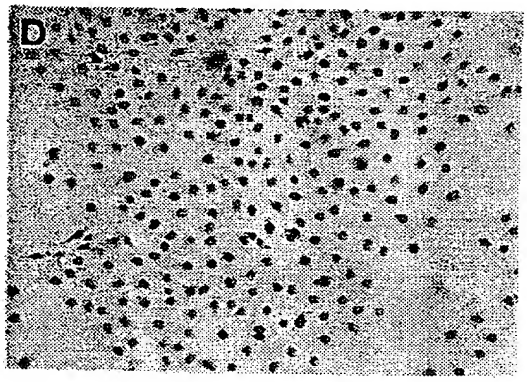

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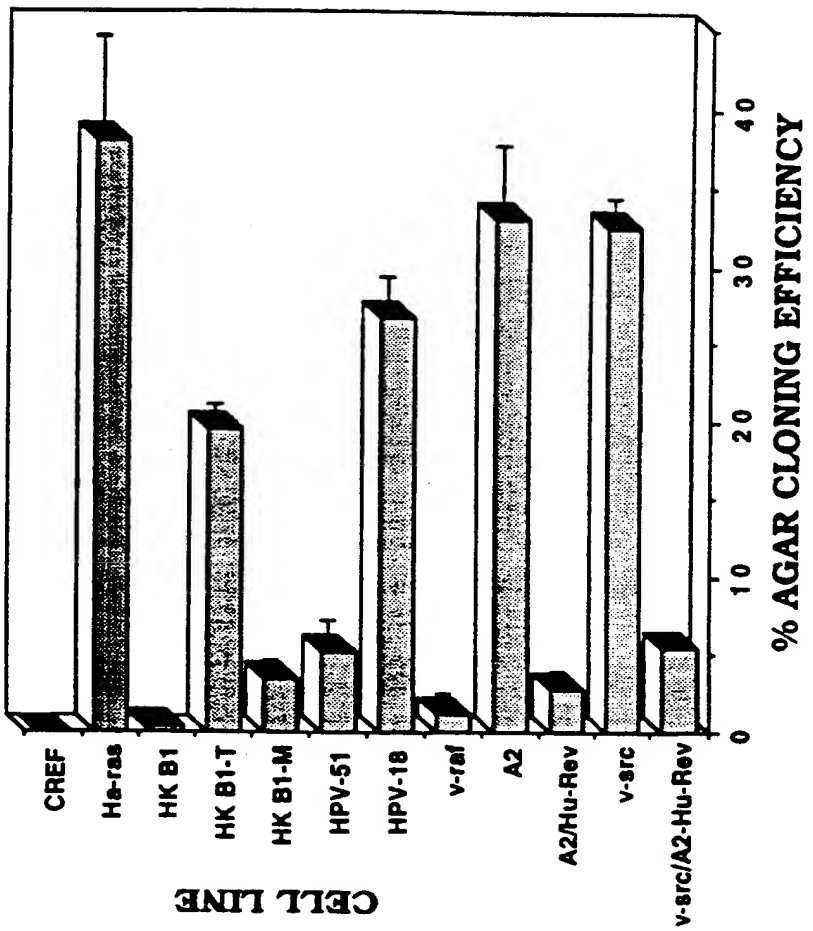
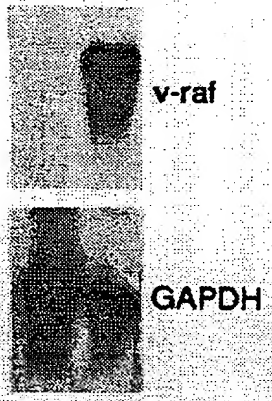


FIGURE 4A



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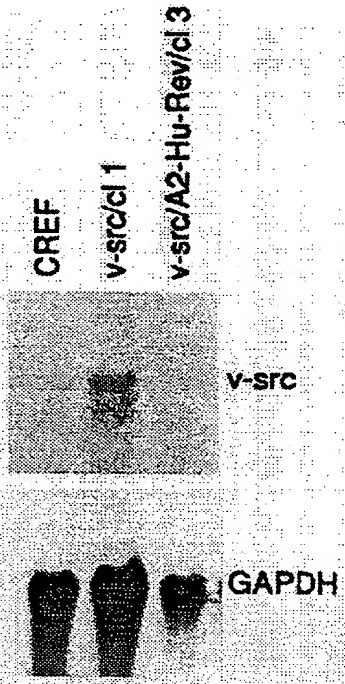

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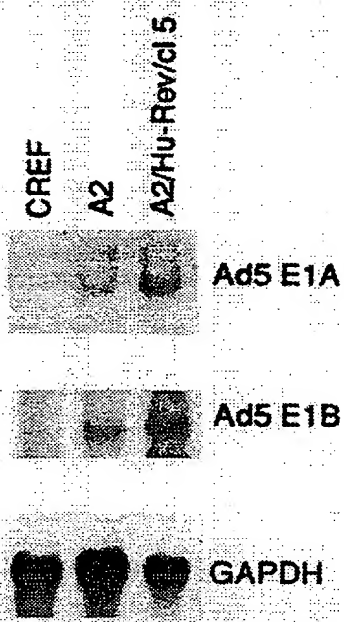

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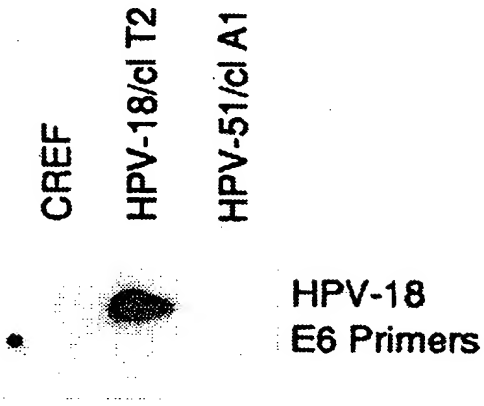


FIGURE 5B

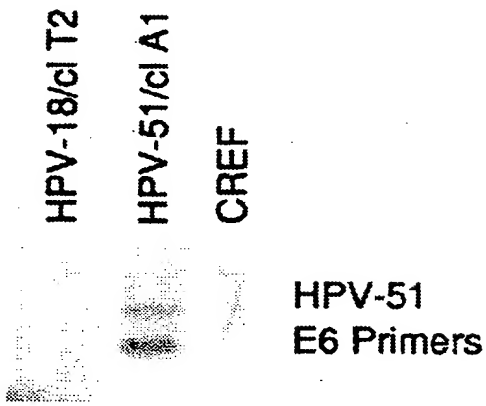


FIGURE 6A

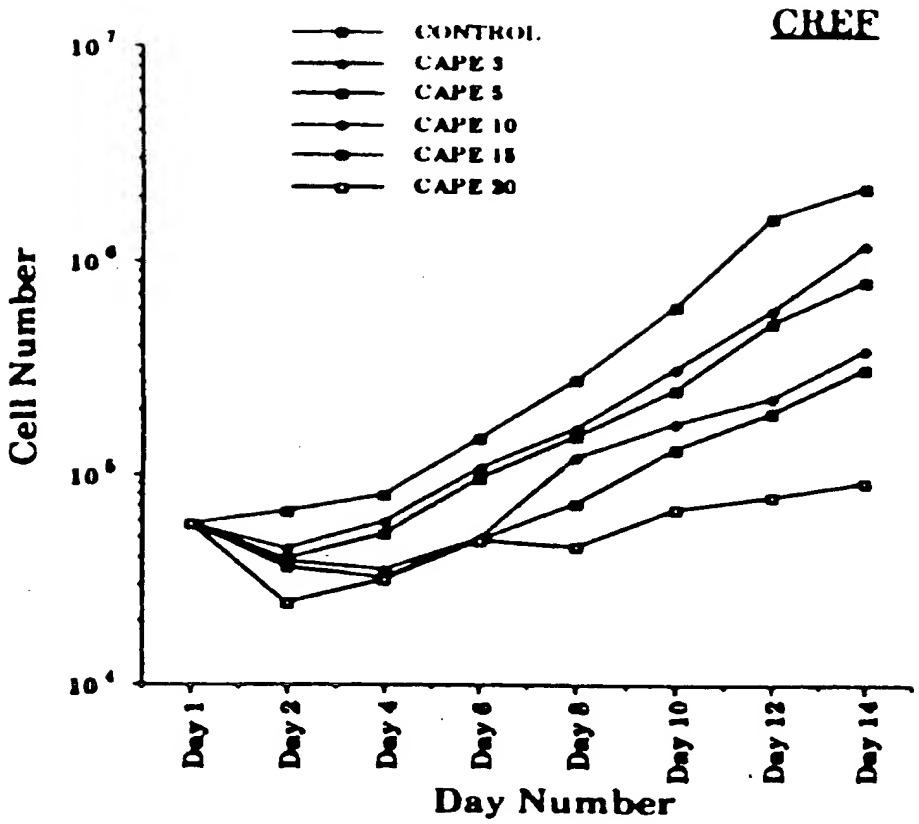

CREF
CONTROL
CAPE 3
CAPE 5
CAPE 10
CAPE 15
CAPE 20
Cell Number
10 7
10 6
10 5
10 4
Day 1
Day 2
Day 4
Day 6
Day 8
Day 10
Day 12
Day 14
Day Number

FIGURE 6B

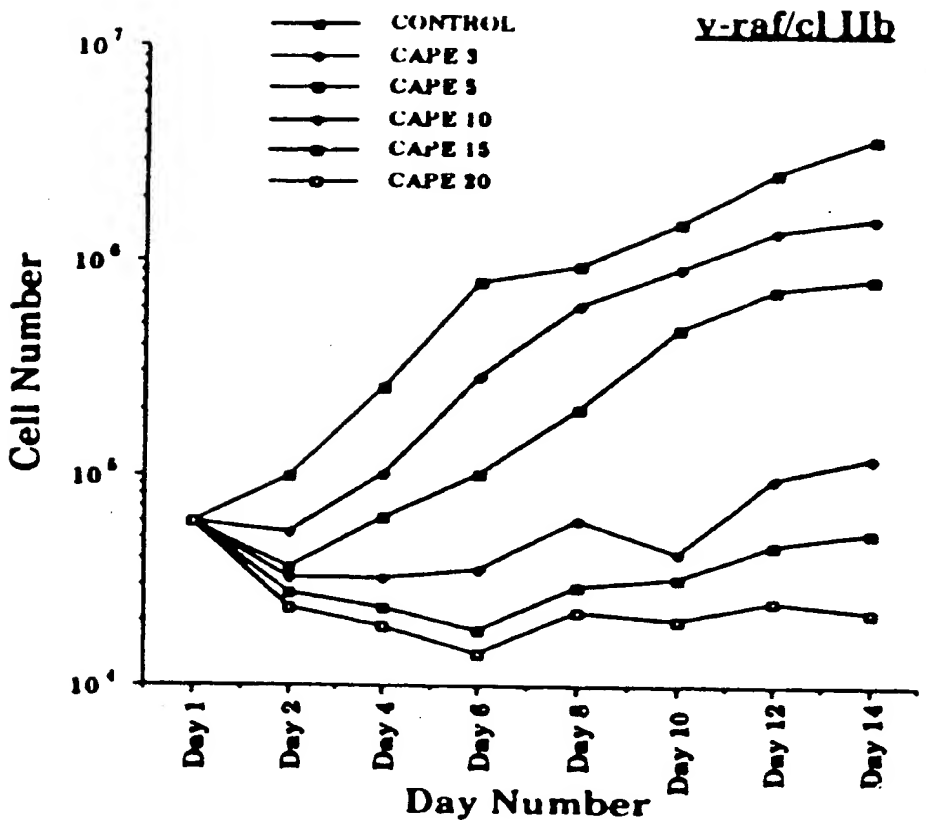


FIGURE 6C

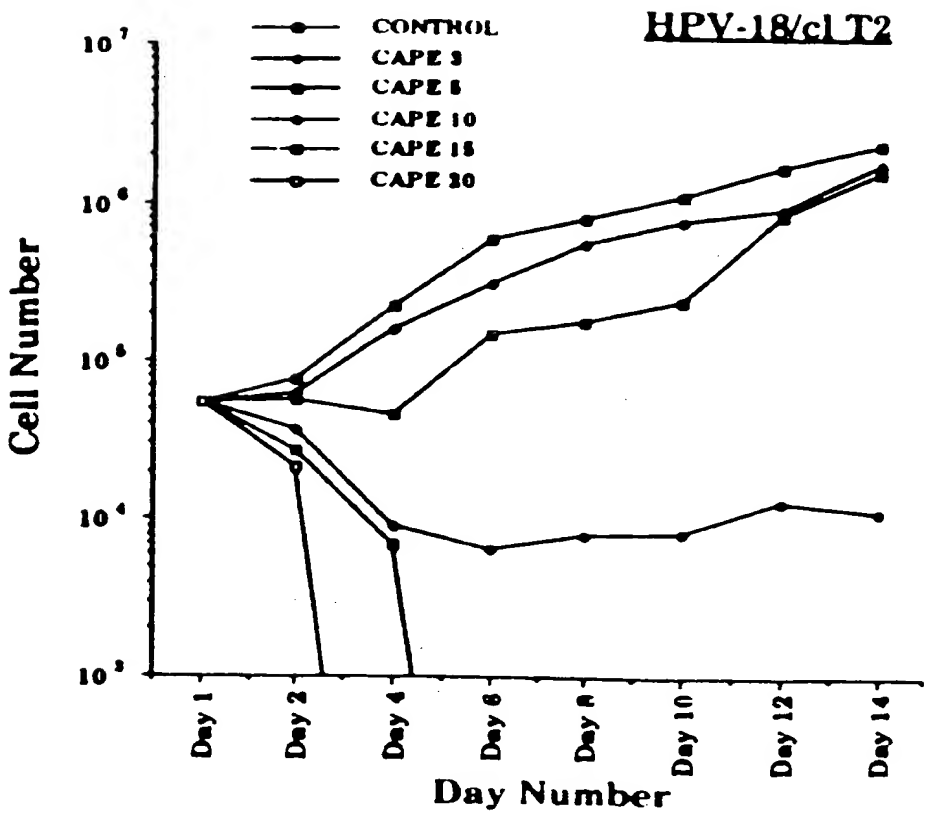


FIGURE 6D

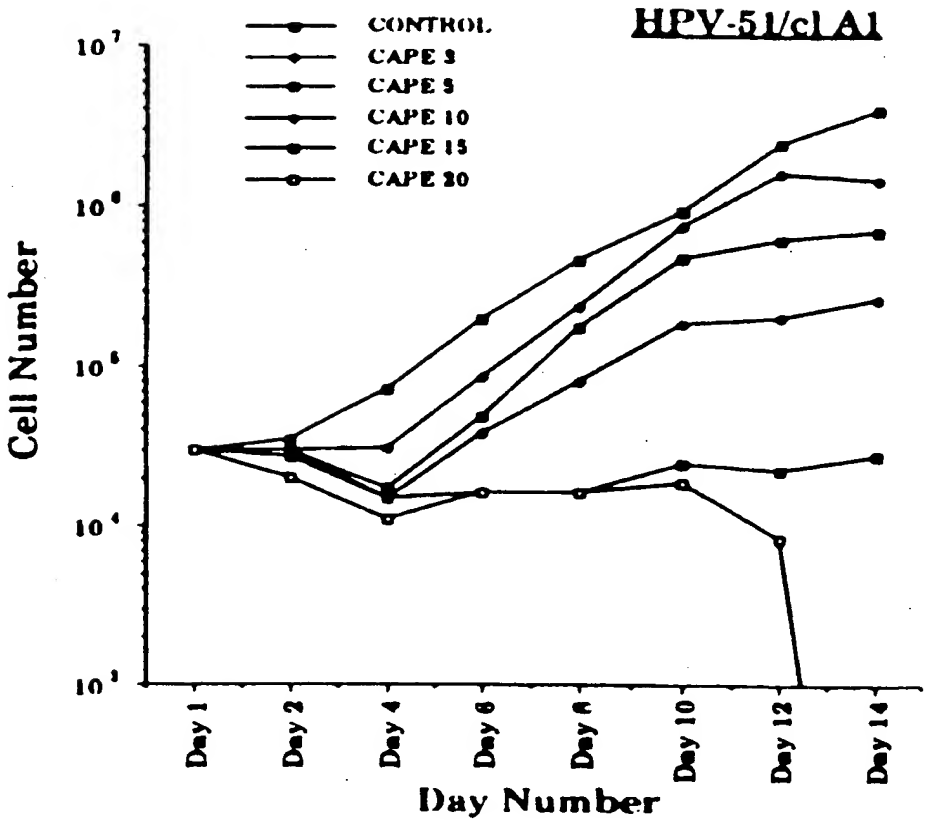


FIGURE 7A

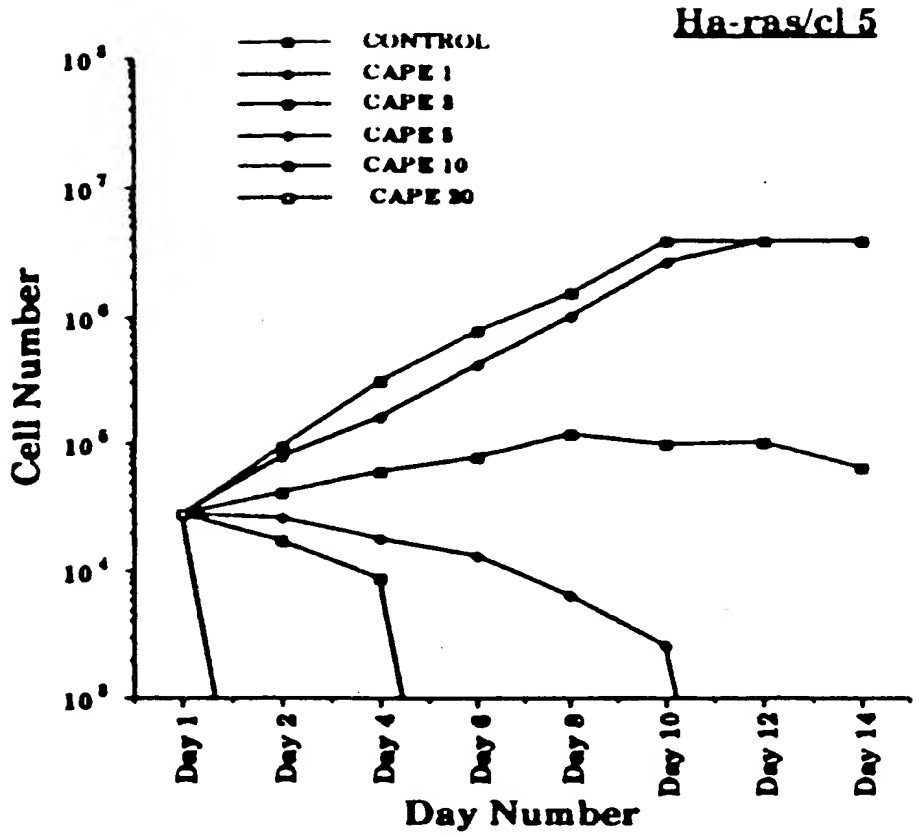

Ha-ras/cl 5
CONTROL
CAPE 1
CAPE 3
CAPE 5
CAPE 10
CAPE 20
Cell Number
Day Number
Day 1
Day 2
Day 4
Day 6
Day 8
Day 10
Day 12
Day 14

FIGURE 7C

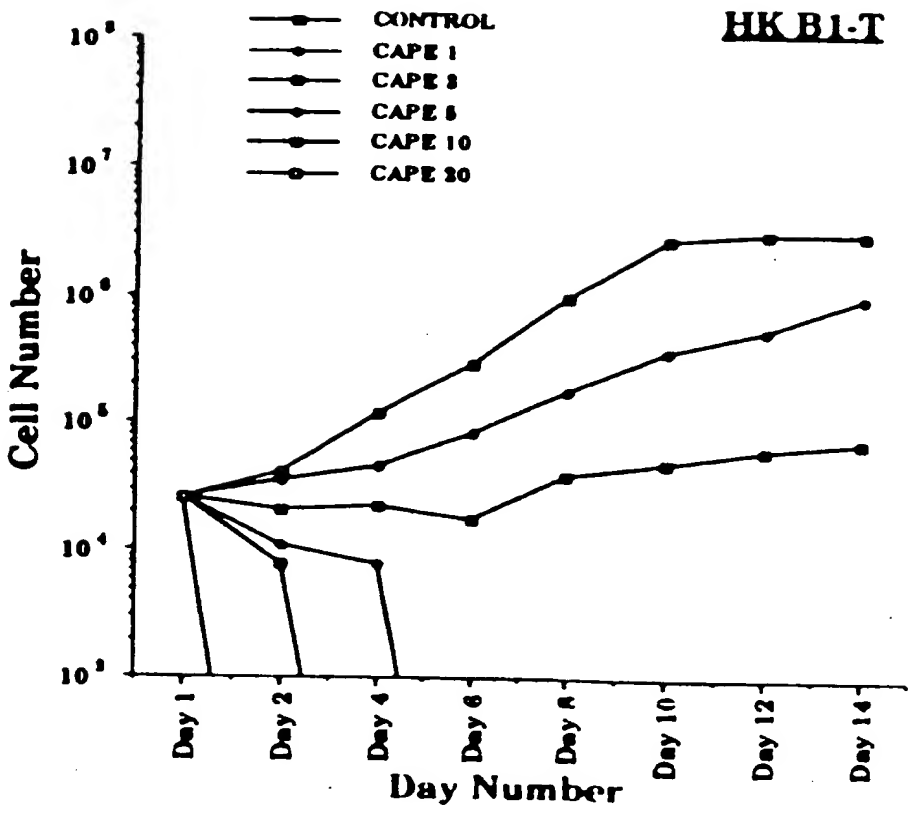


FIGURE 7C

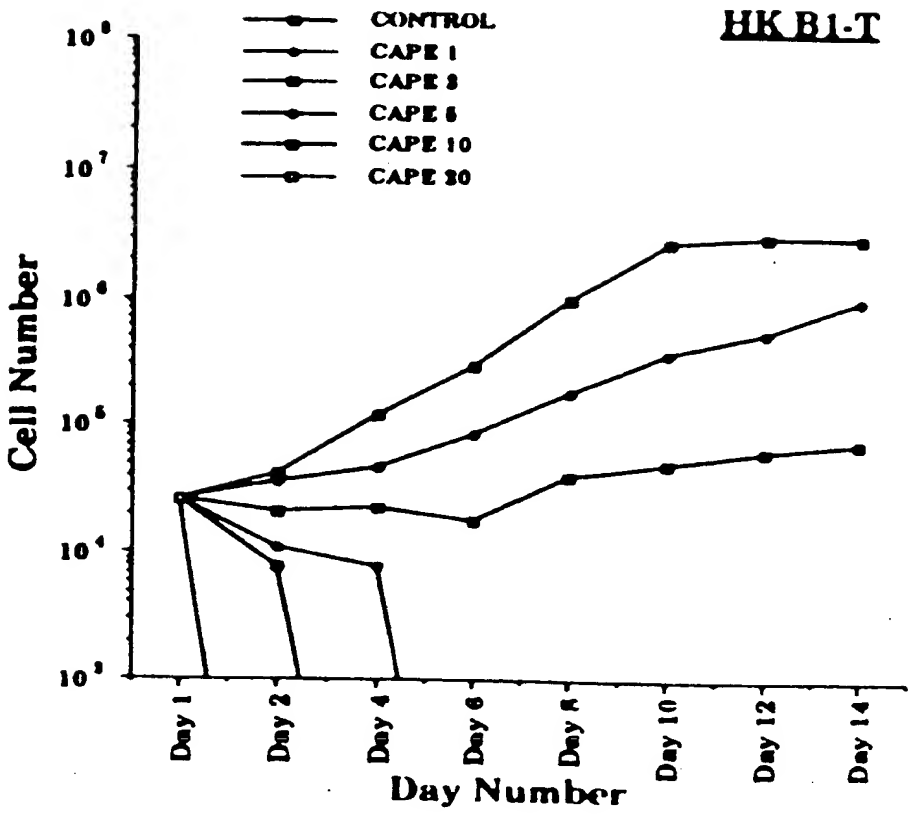


FIGURE 7D

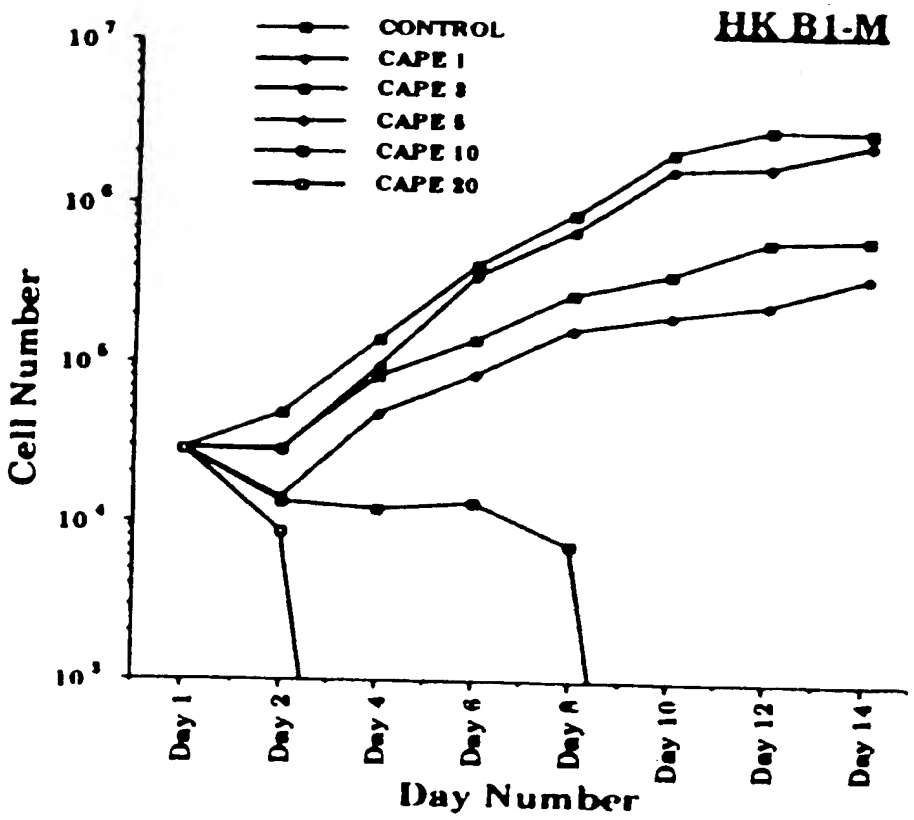


FIGURE 8A

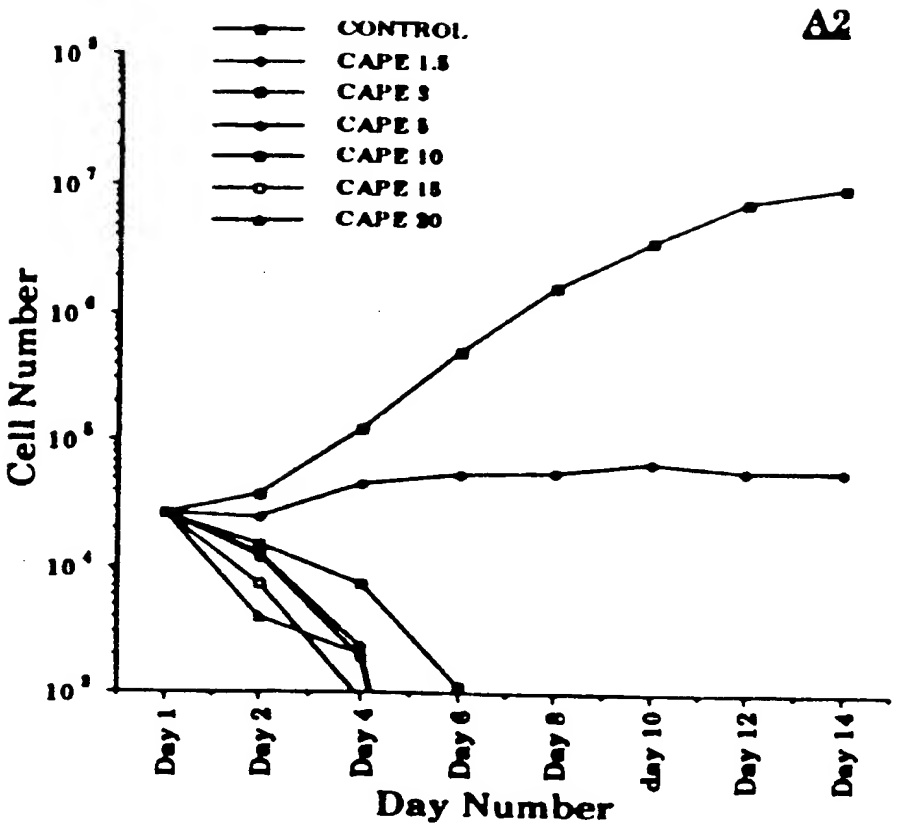


FIGURE 8B

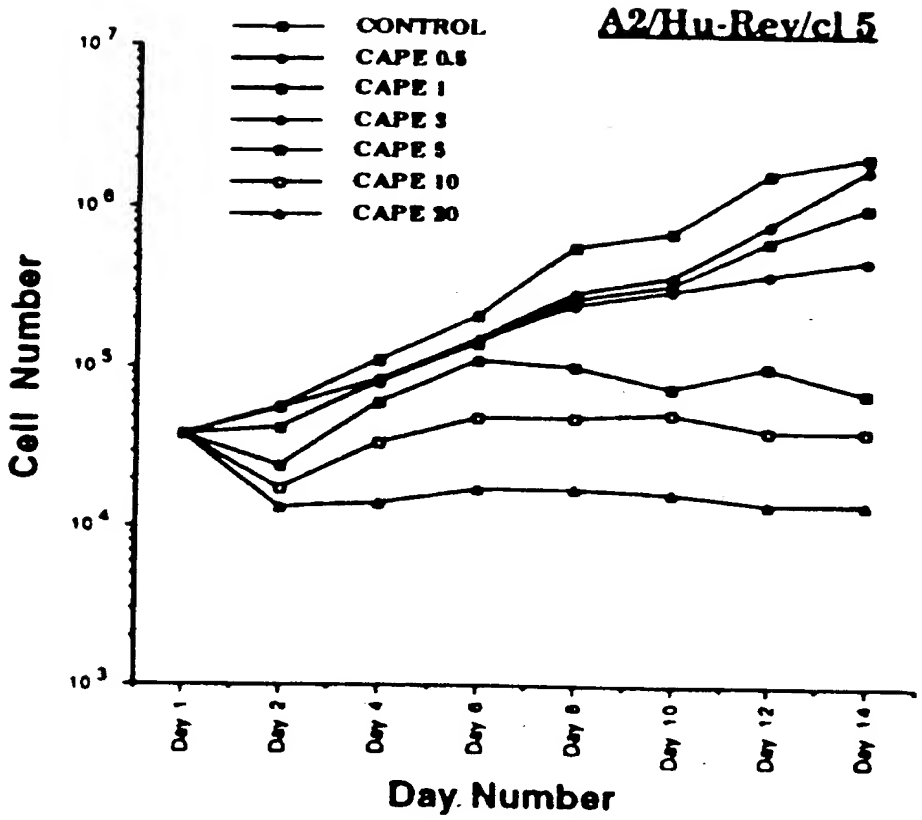


FIGURE 8C

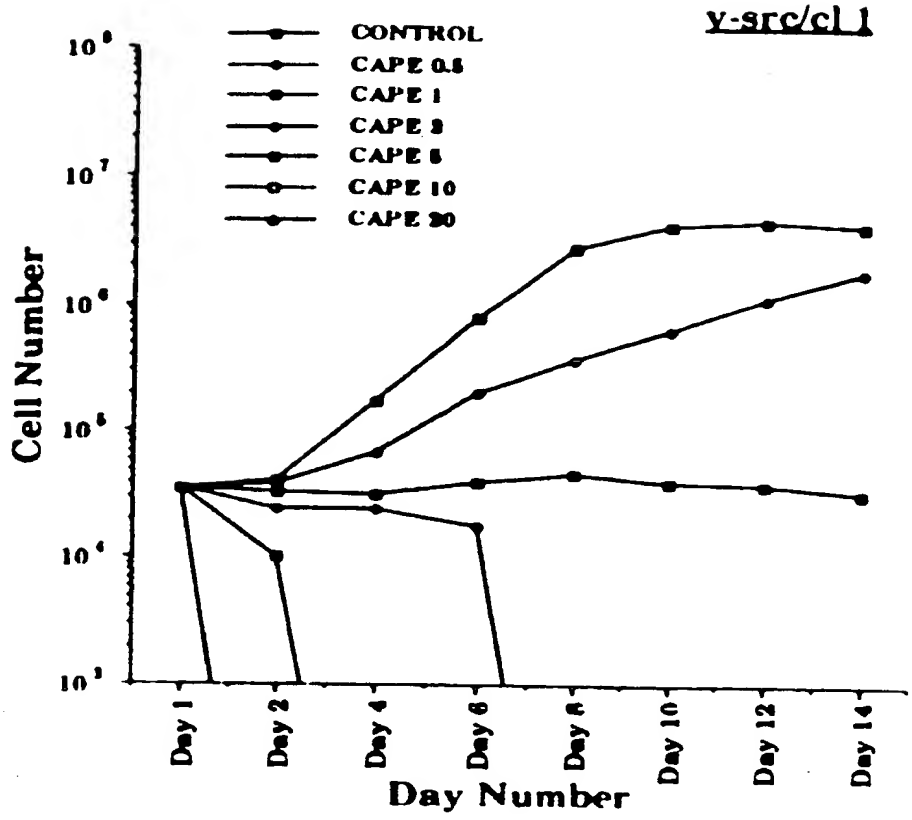


FIGURE 8D

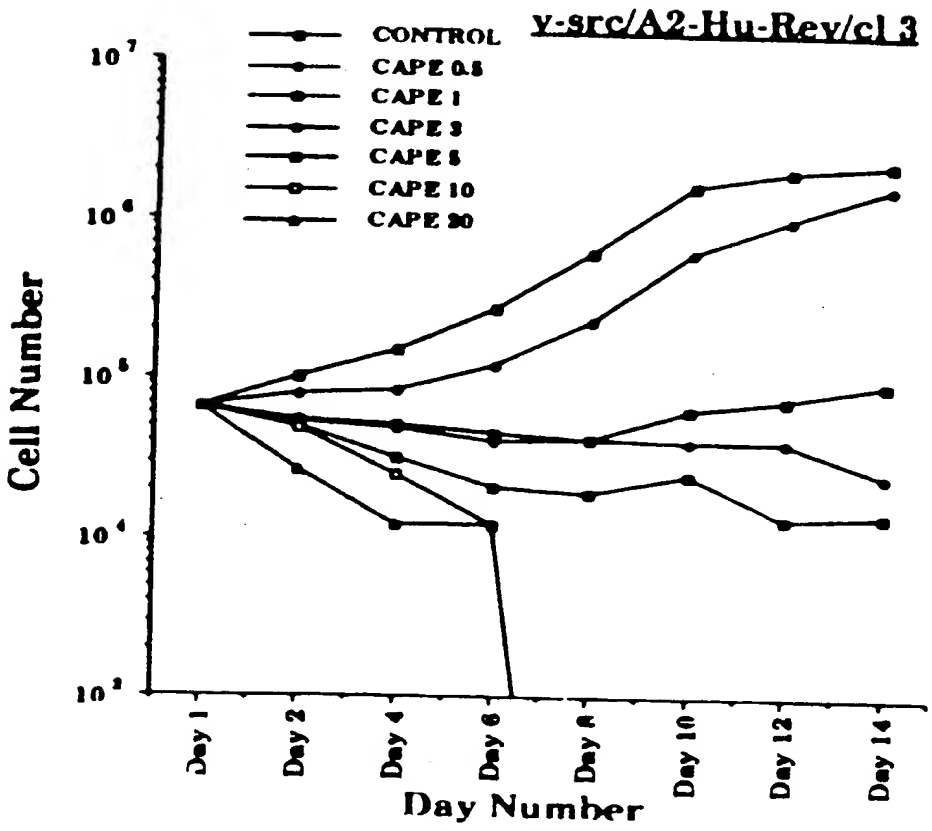


FIGURE 9

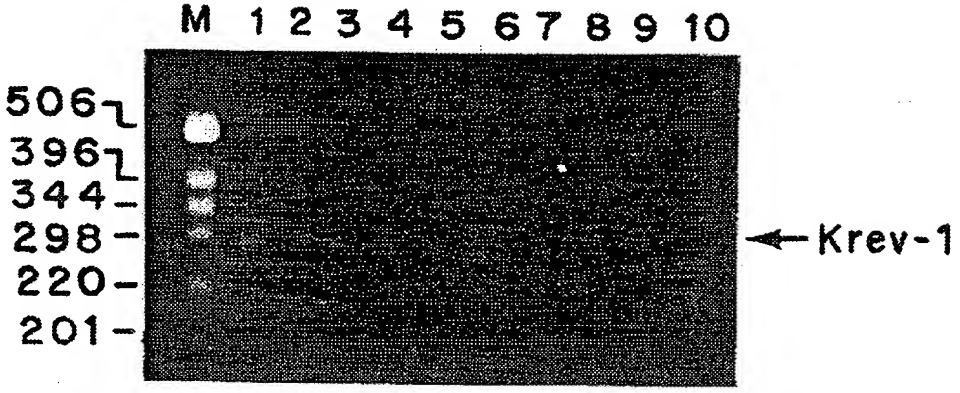


FIGURE 10

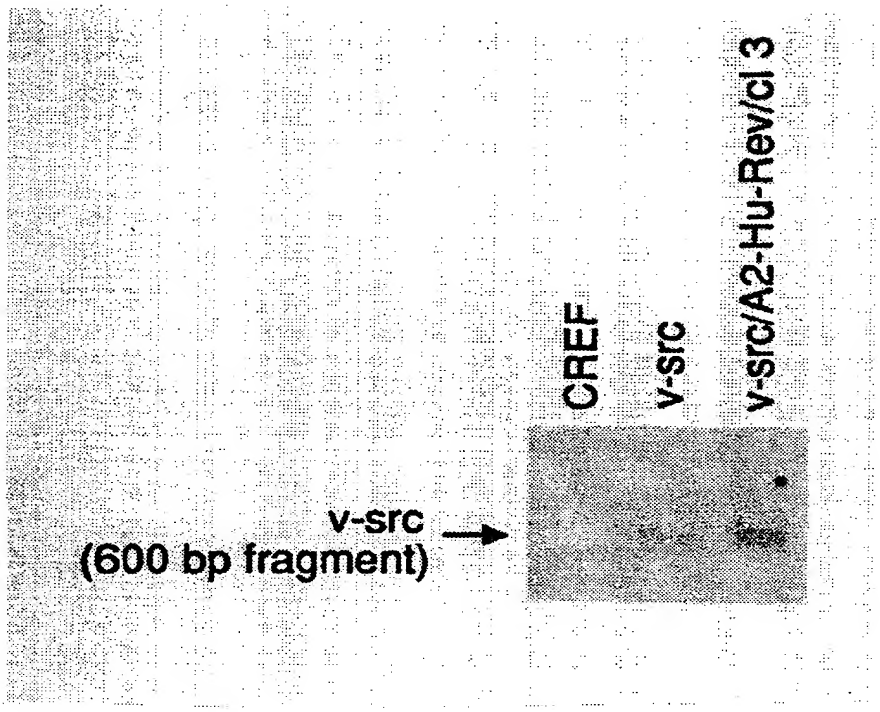


FIGURE 11

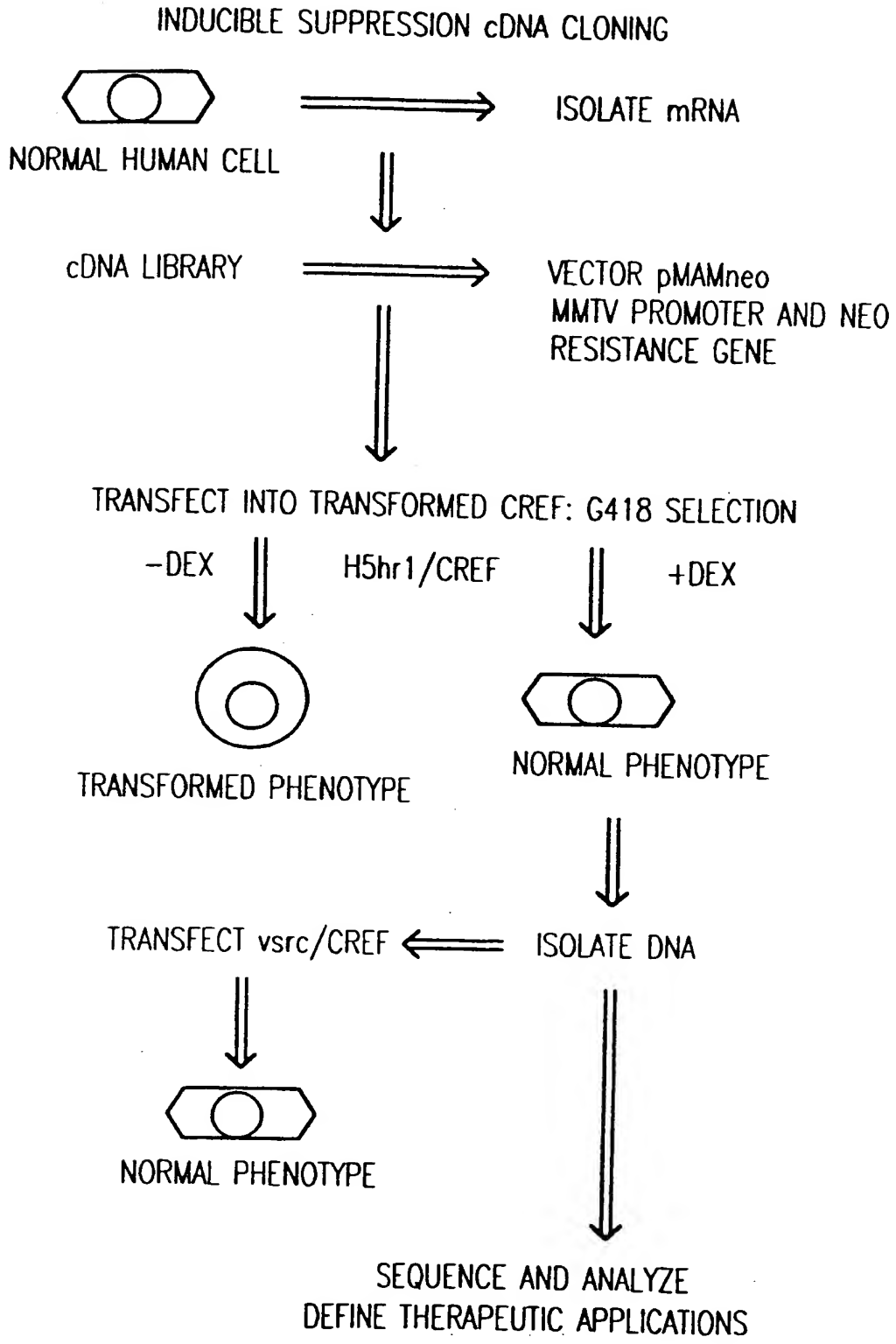


FIGURE 12

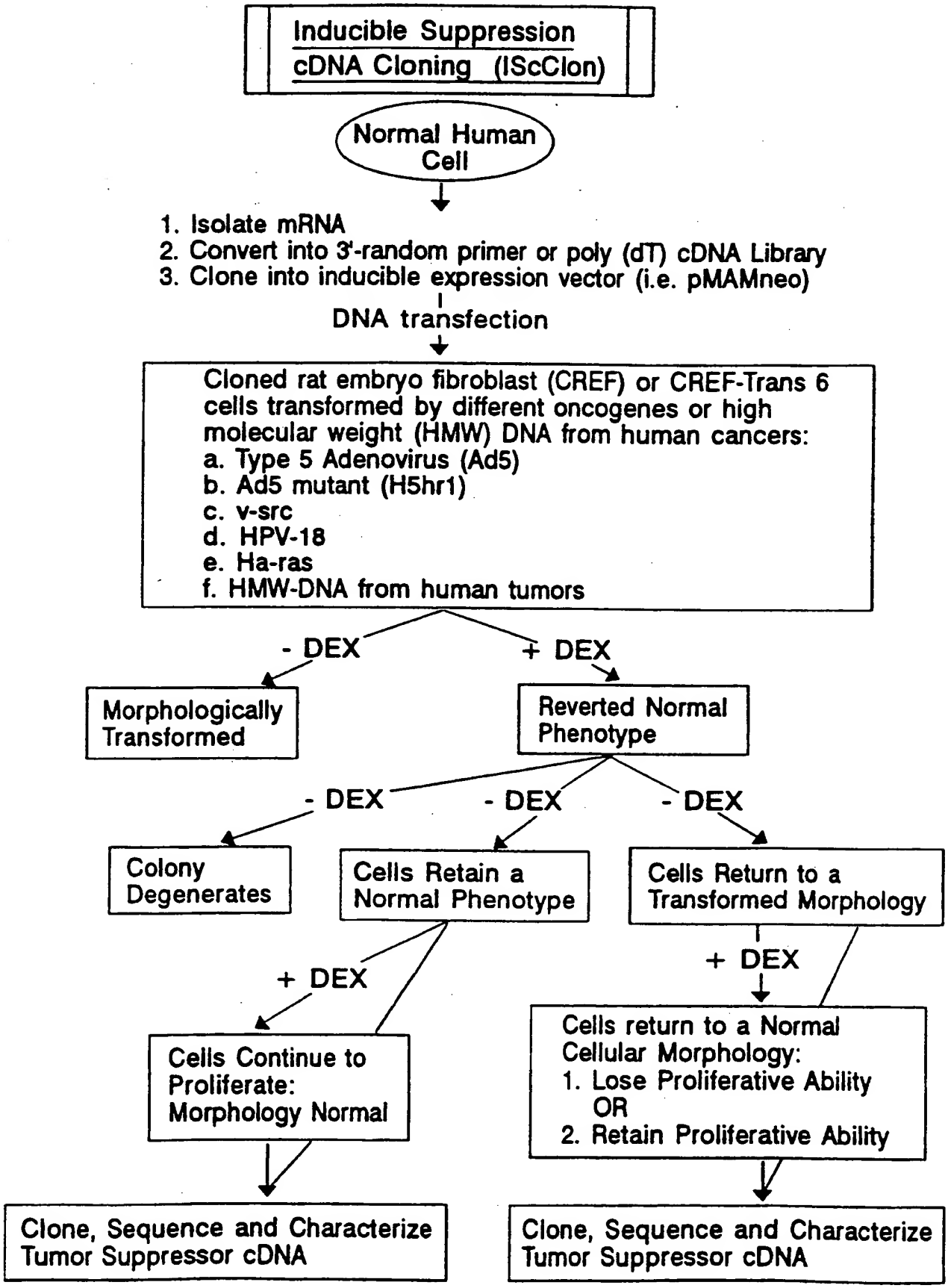


FIGURE 13

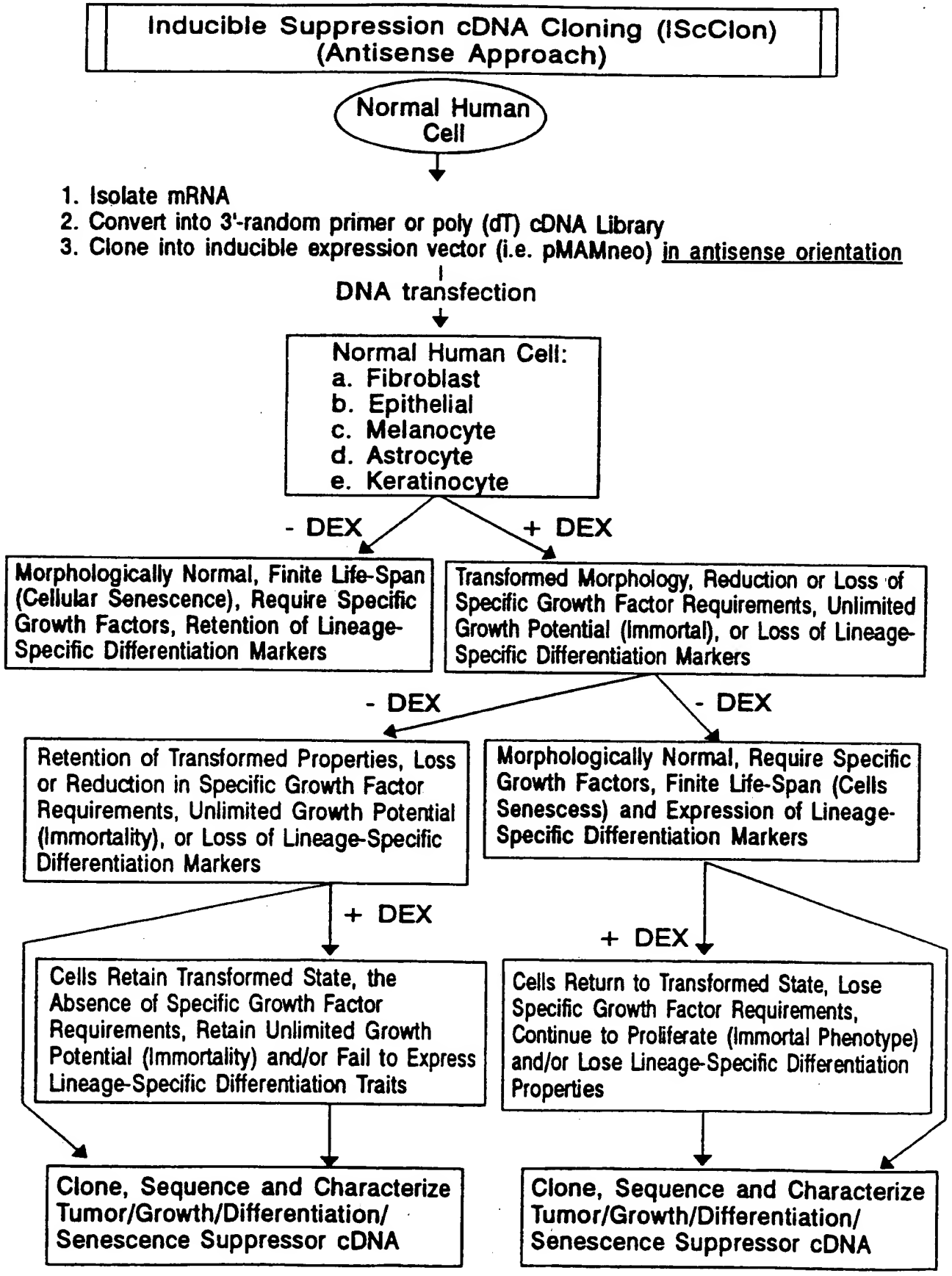
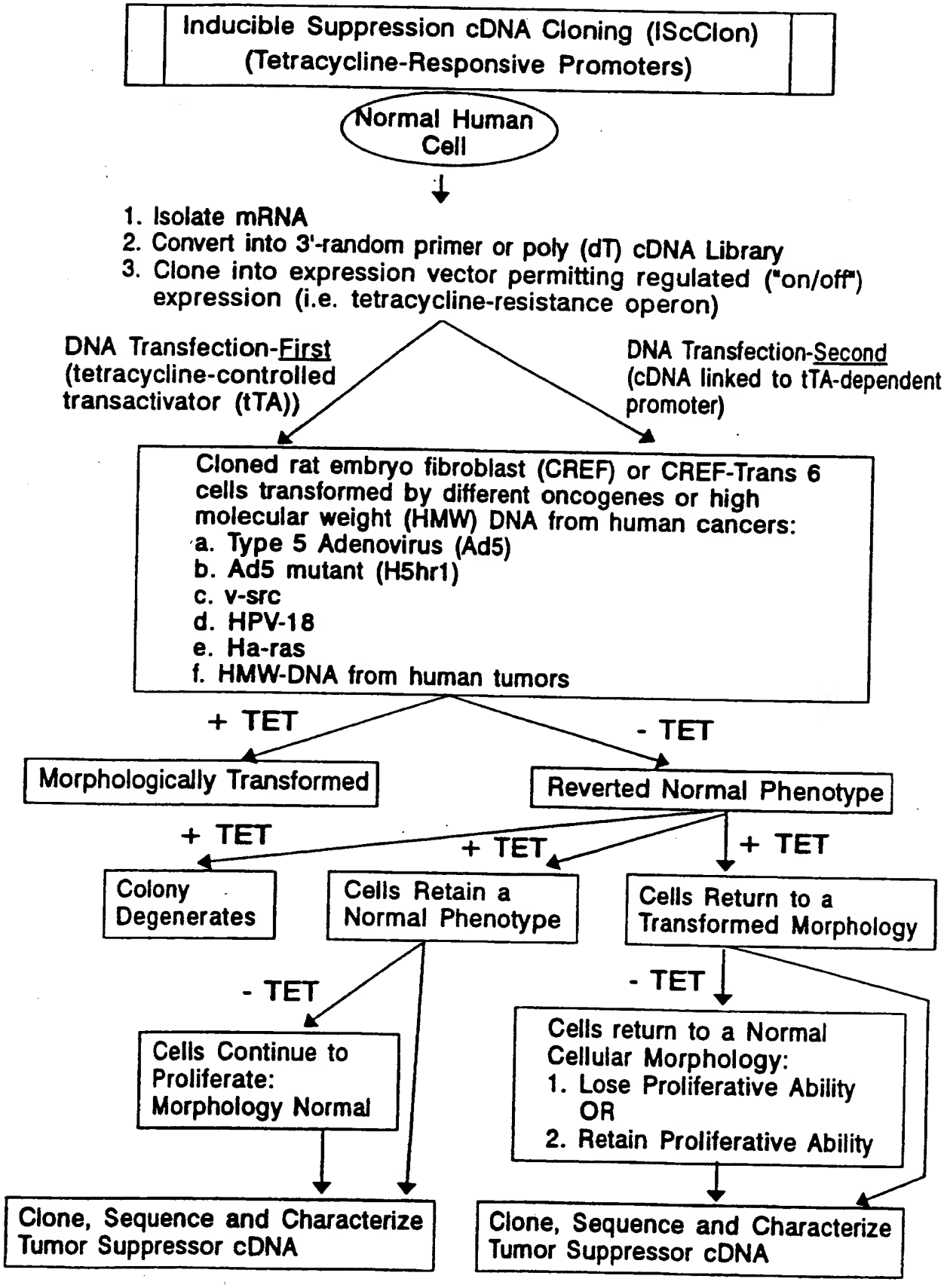

Inducible Suppression cDNA Cloning (IScClon)
(Antisense Approach)
Normal Human Cell
1. Isolate mRNA
2. Convert into 3'-random primer or poly (dT) cDNA Library
3. Clone into inducible expression vector (i.e. pMAMneo) in antisense orientation
DNA transfection
Normal Human Cell:
a. Fibroblast
b. Epithelial
c. Melanocyte
d. Astrocyte
e. Keratinocyte
- DEX
+ DEX
Morphologically Normal, Finite Life-Span (Cellular Senescence), Require Specific Growth Factors, Retention of Lineage-Specific Differentiation Markers
Transformed Morphology, Reduction or Loss of Specific Growth Factor Requirements, Unlimited Growth Potential (Immortal), or Loss of Lineage-Specific Differentiation Markers
- DEX
- DEX
Retention of Transformed Properties, Loss or Reduction in Specific Growth Factor Requirements, Unlimited Growth Potential (Immortality), or Loss of Lineage-Specific Differentiation Markers
Morphologically Normal, Require Specific Growth Factors, Finite Life-Span (Cells Senescess) and Expression of Lineage-Specific Differentiation Markers
+ DEX
+ DEX
Cells Retain Transformed State, the Absence of Specific Growth Factor Requirements, Retain Unlimited Growth Potential (Immortality) and/or Fail to Express Lineage-Specific Differentiation Traits
Cells Return to Transformed State, Lose Specific Growth Factor Requirements, Continue to Proliferate (Immortal Phenotype) and/or Lose Lineage-Specific Differentiation Properties
Clone, Sequence and Characterize Tumor/Growth/Differentiation/ Senescence Suppressor cDNA
Clone, Sequence and Characterize Tumor/Growth/Differentiation/ Senescence Suppressor cDNA

FIGURE 14



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07738

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68, C07H 21/02
US CL : 435/6; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 56, issued January 1989, Kitayama et al, "A ras-related gene with transformation suppressor activity", pages 77-83, see pages 77-80.	1-20 and 25-29
Y	Nature, Volume 294, issued 19 November 1981, Lee et al, "Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids", pages 228-232, see entire document.	1-20 and 25-29
Y	Science, Volume 242, issued 16 December 1988, Huang et al, "Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells", pages 1563-1566, see entire document.	25-29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
29 AUGUST 1995	13 OCT 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer D FIELDS ETHAN WHISENANT Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

In..ational application No.
PCT/US95/07738

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Science, U.S.A., Volume 86, issued January 1989, Noda et al, "Detection of genes with a potential for suppressing the transformed phenotype associated with activated ras genes", pages 162-166, see entire document.	1-20 and 25-29
Y	Proceedings of the National Academy of Science, U.S.A., Volume 80, Number 18, issued September 1983, Noda et al, "Flat revertants isolated from Kirsten sarcoma virus-transformed cells are resistant to the action of specific oncogenes", pages 5602-5606, see entire document.	1-20 and 25-29
Y	Proceedings of the National Acadeny of Science, U.S.A., Volume 79, issued June 1982, Fisher et al, "Analysis of type 5 adenovirus transformation with a cloned rat embryo cell line (CREF)", pages 3527-3531, see pages 3527-3530.	1-20 and 25-29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07738

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-20 and 25-29

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Int. ..ational application No.
PCT/US95/07738

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAPLUS, MEDLINE
search terms: tumor suppressor gene(s), transformation, inducer, inducible promoter,cDNA, revertant(s), transformation suppressor gene(s)

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s)1-20 and 25-29, drawn to a method of identifying a tumor suppressor gene.
Group II, claim(s) 21, drawn to a virus which contains a tumor suppressor gene.
Group III, claim(s) 22-23, drawn to a polypeptide encoded by the tumor suppressor gene and the antibody capable of binding to the polypeptide.
Group IV, claim 24, drawn to a non-human mammal whose germ cells or somatic cells contain a recombinant tumor suppressor gene.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group II differs from Group I because the virus containing a tumor suppressor gene is not a product of the method nor is it required to perform the method of Group I.
The inventions listed as Groups I and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group III the polypeptide produced by the tumor suppressor gene and the antibody against this polypeptide are not a product of the method. Also, the method does not require the polypeptide or the antiibody to be performed. Unity of invention is lacking.

The inventions listed as Groups I and IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group IV the non-human mammal whose cells contain a recombinant tumor suppressor gene are not required to perform the method of Group I nor is the non-human mammal an end product of the method of Group I.

The inventions listed as Groups II and III do not relate to a single inventive concept under PCT Rule 13.1 because,underPCTRule 13.2, they lack the same or corresponding special technical features for the following reasons: the virus containing the tumor suppressor gene is not required to isolate the polypeptide and its corresponding antibody. In addition, the polypeptide and its corresponding antibody are not required to make the virus of claim 20.
The inventions listed as Groups II and IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding specialtechnicalfeatures for the following reasons: the virus containining the tumor suppressor gene is not required to produce the non-human mammal. Likewise the virus is not product of the non-human mammal.

The inventions listed as Groups III and IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the polypeptide and its corresponding antibody are not produced by the non-human mammals of Claim 24 nor are the polypeptide and antibody required to produce these animals. They are not linked by a special technical feature.

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